PATENT
Docket No.: **19603/3357 (CRF D-1595G)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Barany et al.	Examiner: To Be Assigned
Serial No.	:	09/986,527	
Cfrm. No.	:	To be Assigned	Art Unit: To Be Assigned
Filed	:	November 9, 2001	
For	:	DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING THE LIGASE DETECTION REACTION WITH ADDRESSABLE ARRAYS	

DECLARATION OF GREGORY W. SISKIND UNDER 37 CFR § 1.608(b)

Commissioner of Patents
Washington, D.C. 20231

Dear Sir:

I, GREGORY W. SISKIND, pursuant to 37 CFR § 1.608(b), declare:

1. I received a B.A. in 1955 from Cornell University and an M.D. from the New York University School of Medicine in 1959.

2. I am the Associate Dean/Research Compliance at the Joan and Sanford Weill I. Medical College of Cornell University, New York, New York ("Cornell Medical College").

3. As Associate Dean at Cornell Medical College, my responsibilities have included the submission of federal grant applications by Cornell Medical College faculty members as well as the administration of federal grants awarded to Cornell Medical College faculty members. I have also been responsible for maintaining Cornell Medical College's files of grant applications submitted and grants awarded.

4. Cornell Medical College's files of grant applications submitted include a grant application, entitled "New Methods for Cancer Detection", ("Grant Application") which was submitted to the National Cancer Institute, U.S. Department of Health and Human

Services ("NCI") by, amongst others, Francis Barany of Cornell Medical College on February 4, 1994. This application describes 5 projects, including Project 5, entitled "Design and Synthesis of DNA and PNA Arrays". A copy of relevant portions of the Grant Application, including the Project 5 description are attached hereto at Appendix 1.

5. In accordance with my above-noted responsibilities as Associate Dean at Cornell Medical College, on February 4, 1994, I signed the certification at section 18 for the Grant Application on behalf of Cornell Medical College (Appendix 1 at p. 1).

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12/18/01

Gregory W. Siskind

Appendix 1

(To Declaration of Gregory W. Siskind under 37 CFR § 1.608(b))

AA

Form Approved Through 6/30/94
OMB No. 0925-0001

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PROGRAM PUBLIC HEALTH SERVICE
PROJECT **GRANT APPLICATION**
Follow instructions carefully. Type in the unshaded areas only. Type density must be 10 c.p.i.

LEAVE BLANK FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.)
NEW METHODS FOR CANCER DETECTION

2a. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☒ NO ☐ YES *(If "YES," state number and title)*
Number: Title: CONFIDENTIAL INFORMATION

2b. TYPE OF GRANT PROGRAM P01

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*
BARANY, FRANCIS

3b. DEGREE(S)
PH.D.

3c. SOCIAL SECURITY NO.
REDACTED

3d. POSITION TITLE
ASSOCIATE PROFESSOR

3e. MAILING ADDRESS *(Street, city, state, zip code)*
CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
MICROBIOLOGY

3g. MAJOR SUBDIVISION
CORNELL UNIV. MEDICAL COLLEGE

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 212 746-6509
FAX: 212 746-8587

BITNET/INTERNET ADDRESS BARANY@CUMC.CORNELL.EDU

4. HUMAN SUBJECTS
X 4a. NO ☐ YES If "Yes," exemption no. or IRB approval date 4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS
X 5a. NO ☐ YES If "Yes," IACUC approval date 5b. Animal welfare assurance no.

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD	
From (MMDDYY)	Through (MMDDYY)	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
120194	113099	$971,041	$1,224,862	$5,295,869	$6,995,293

9. PERFORMANCE SITES *(Organizations and addresses)*
CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

10. INVENTIONS AND PATENTS *(Competing continuation application only)*
☐ NO ☐ YES If "YES," ☐ Previously reported ☐ Not previously reported

11. NAME OF APPLICANT ORGANIZATION
CORNELL UNIVERSITY MEDICAL COLLEGE
ADDRESS 1300 YORK AVENUE
NEW YORK, NY 10021

12. TYPE OF ORGANIZATION
☐ Public: *Specify* ☐ *Federal* ☐ *State* ☐ *Local*
☒ Private Nonprofit
☐ Forprofit *(General)* ☐ Forprofit *(Small Business)*

13. ENTITY IDENTIFICATION NUMBER
1131623978A1

Congressional District
14

14. BIOMEDICAL RESEARCH SUPPORT GRANT CREDIT
Code: 01 Identification: SCHOOL OF MEDICINE

15. NAME OF ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
PHILIP V. GIUCA
TELEPHONE 212 746-6036
FAX 212 746-8745
TITLE SENIOR ASSOCIATE DEAN
ADDRESS CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

BITNET/INTERNET ADDRESS QMCUMC.MAIL.CORNELL.EDU

16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
GREGORY W. SISKIND
TELEPHONE 212 746-6020
FAX 212 746-8745
TITLE ASSOCIATE DEAN
ADDRESS CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

BITNET/INTERNET ADDRESS QMCUMC.MAIL.CORNELL.EDU

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
Dr F. Barany

DATE
2/4/94

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 16. *(In ink. "Per" signature not acceptable)*
Gregory W. Siskind

DATE
2/4/94

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. DO NOT EXCEED THE SPACE PROVIDED.

The long range objective of this proposal is to develop sensitive and specific approaches to the detection and simultaneous identification of cancer-related, genetic alterations. Mutations and genetic aberrations have been implicated, at various steps, in the etiology and biology of tumors. Inherited mutations account for the predisposition to cancer in some families. Somatic mutations in tumor suppressor genes, oncogene amplification and viral DNA sequences have been found in cancers as well. However, the clinical use of these discoveries and research into their clinical significance has been slowed by the laborious processes by which they are detected. To apply these discoveries and explore the interactions of multiple genetic alterations, we urgently need a new technology, which is capable of being automated and has the power to detect any of a vast number of mutations.

In response to the urgent need for new methods of mutation detection, we have assembled a team of investigators whose expertise will be directed toward innovative solutions to this problem. The collaborative nature of the scientific and organizational infrastructure will facilitate the attainment of the projects' specific aims and objectives.

The specific aims of the five projects in this proposal are to: (i) develop a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) system for the detection of inherited mutations in germline DNA and somatic mutations in tumors; (ii) develop a ligase detection reaction/ polymerase chain reaction (LDR/PCR) system for detecting gene amplifications and deletions in tumors; (iii) develop a PCR/restriction enzyme/LDR (PCR/RE/LDR) system for detecting and identifying mutations in rare cancer cells at a sensitivity of 1 in 10^6 or 1 in 10^7 by removing normal DNA sequences and selectively amplifying cancer mutations; (iv) design and synthesize nucleotide analogues for converting specific DNA sequences into restriction endonuclease recognition sites for PCR/RE/LDR mutation detection; (v) engineer a thermostable ligase with greater fidelity to enhance LDR and LCR specificity; (vi) design and synthesis oligonucleotide or peptide nucleic acid (PNA) addressable arrays for the simultaneous detection of multiplex LDR and LCR products; and (vii) explore the ability of these technologies to further our understanding and clinical management of lung, colon, breast and cervical cancers.

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. *Use continuation pages as needed* to provide the required information in the format shown below on *all* individuals participating in the project.

Name AGGARWAL, Aneel	Degree(s) Ph.D.	Social Security # REDACTED	
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Co-investigator	
Organization College of Physicians & Surgeons of Columbia University		Department Biochem & Biophys	
Name AHNEN, Dennis	Degree(s) M.D.	Social Security # REDACTED	
Position Title VA Clinical Investigator /Associate Professor	D.O.B. REDACTED	Role on Project Collaborator	
Organization Veterans Affairs Medical Center & Univ. Colorado School Of Medicine		Department Medicine	
Name BARANY, Francis	Degree(s) Ph.D.	Social Security # REDACTED	
Position Title Associate Professor	D.O.B. REDACTED	Role on Project Prin. Investig.	
Organization Cornell Univeristy Medical College		Department Microbiology	
Name BARANY, George	Degree(s) Ph. D.	Social Security # REDACTED	
Position Title Professor	D.O.B. REDACTED	Role on Project Co-investigator	
Organization University of Minnesota		Department Chemistry	
Name BATT,Carl	Degree(s) Ph.D.	Social Security # REDACTED	
Position Title Associate Professor	D.O.B. REDACTED	Role on Project Collaborator	
Organization Cornell University		Department Food Science	
Name BERGSTROM, Donald	Degree(s) Ph.D.	Social Security # REDACTED	
Position Title Professor	D.O.B. REDACTED	Role on Project Co-investigator	
Organization Purdue Univ. School of Pharmacy &Pharm. Sciences		Department Medicinal Chem.	
Name BUNK, Michael	Degree(s) Ph.D.	Social Security # REDACTED	
Position Title Director, Research Management	D.O.B. REDACTED	Role on Project Administrator	
Organization Strang Cancer Prevention Center		Department Res. Management	

Name COOK, Ronald	Degree(s) Ph. D.	Social Security # REDACTED
Position Title President/Chief Technical Officer	D.O.B. REDACTED	Role on Project Collaborator
Organization Siris Laboratories		Department Chemistry
Name COTHERN, Melissa	Degree(s) B.S.	Social Security # REDACTED
Position Title Graduate Student	D.O.B. REDACTED	Role on Project Synthetic Chem.
Organization Louisiana State University		Department Chemistry
Name COULL, James	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Group Manager of Specialty Chemistry	D.O.B. REDACTED	Role on Project Collaborator
Organization Millipore Corporation		Department Specialty Chemistry
Name COURVALIN, Patrice	Degree(s) M. D.	Social Security # not applicable
Position Title Professor, Associate Chairman	D.O.B. REDACTED	Role on Project Collaborator
Organization Institut Pasteur		Department Bacteriol. & Mycol.
Name DAY, Darren	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Research Associate	D.O.B. REDACTED	Role on Project
Organization Cornell Univeristy Medical College		Department Microbiology
Name FISHMAN, Jack	Degree(s) Ph. D.	Social Security # REDACTED
Position Title Director of Research	D.O.B. REDACTED	Role on Project Collaborator
Organization Strang Cancer Research Lab		Department Horm. Carcinogens
Name FRANKLIN, Wilbur	Degree(s) M.D.	Social Security # REDACTED
Position Title Professor, Director of Tissue Bank	D.O.B. REDACTED	Role on Project Collaborator
Organization University of Colorado, School of Medicine		Department Pathology
Name FRIEND, Steven	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Harvard Medical School, MGH Cancer Center		Department Cell & Dev. Biol.
Name GELFAND, David	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Director , Core Technology	D.O.B. REDACTED	Role on Project Collaborator
Organization Roche Molecular Systems		Department Protein Core Res.
Name GILES, Aaron	Degree(s) B.S.	Social Security # REDACTED
Position Title Computer Programmer /Analyst II	D.O.B. REDACTED	Role on Project
Organization Cornell University Medical College		Department Acad. Computing
Name GROSSMAN, Larry	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Professor, Associate Chairman	D.O.B. REDACTED	Role on Project Collaborator
Organization Wayne State University, School of Medicine		Department Mol. Biol. & Gen.
Name HACKETT, Neil	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Co-investigator
Organization Cornell Univeristy Medical College		Department Microbiology
Name HAMMER, Robert	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Co-investigator
Organization Louisiana State University		Department Chemistry
Name HERRARA, Vicky	Degree(s) M.D.	Social Security # REDACTED
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Boston University School of Medicine		Department Medicine
Name HOFFMAN, Eric	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Assistant Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization University of Pittsburgh, School of Medicine		Department Mol. Gen. Biochem
Name KENNEDY, Timothy	Degree(s) M.D.	Social Security # REDACTED
Position Title Medical Director	D.O.B. REDACTED	Role on Project Collaborator
Organization Lung Cancer Institut of Colorado		Department Institute
Name KEW, Olen	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Chief of Molecular Virology Section	D.O.B. REDACTED	Role on Project Collaborator
Organization National Center of Infectious Diseases, CDC		Department Viral Division
Name KOLLER, Antje	Degree(s) B.A. equiv.	Social Security # REDACTED
Position Title Technician	D.O.B. REDACTED	Role on Project
Organization Cornell Univeristy Medical College		Department Microbiology
Name KOVACH, John	Degree(s) M.D.	Social Security # REDACTED
Position Title Professor, Chairman, Director NCI Cancer Ctr.	D.O.B. REDACTED	Role on Project Collaborator
Organization Mayo Clinic		Department Oncology

Name LU, Jing	Degree(s) B.A.	Social Security # REDACTED
Position Title Technician	D.O.B. REDACTED	Role on Project
Organization Cornell Univeristy Medical College		Department Microbiology
Name LUBIN, Matthew	Degree(s) M.D.	Social Security # REDACTED
Position Title Director of Medical Genetics	D.O.B. REDACTED	Role on Project Co-investigator
Organization Strang Cancer Prevention Center		Department Medical Genetics
Name LUO, Jianying	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Research Associate	D.O.B. REDACTED	Role on Project
Organization Cornell Univeristy Medical College		Department Microbiology
Name MILLER, Gary	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Associate Professor, Director, Histo. Pathology	D.O.B.	Role on Project Collaborator
Organization Tissue Procurement Core Lad, Univ. of Colorado School of Medicine		Department Pathology
Name NORTHRUP, Allen	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Principal Engineer / Adjunct Assistant Prof.	D.O.B. REDACTED	Role on Project Collaborator
Organization Lawrence Livermore National Lab / U. C. S. F. Medical Center		Department Radiology
Name OSBORNE, Michael	Degree(s) M.D.	Social Security # REDACTED
Position Title Director	D.O.B. REDACTED	Role on Project Collaborator
Organization Strang Cancer Prevention Center		Department Surgery
Name PERSING, David	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Senior Associate Consultant / Assistant Prof.	D.O.B. REDACTED	Role on Project Collaborator
Organization Mayo Foundation		Department Lab. Med.& Pathol.
Name PROUDFOOT, Susan	Degree(s) M.S.H.A.	Social Security # REDACTED
Position Title Executive Director	D.O.B. REDACTED	Role on Project Collaborator
Organization Lung Cancer Institute of Colorado		Department Lung Institute
Name REZNIKOV, Leonid	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Research Fellow	D.O.B. REDACTED	Role on Project
Organization The Children's Hospital & Univ. Colorado Health Sciences Center		Department Pathology
Name RIGAS, Basil	Degree(s) M.D.	Social Security # REDACTED
Position Title Associate Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Cornell University Medical College		Department Microbiol.& Med.
Name ROBERTS, Richard	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Director of Research	D.O.B. REDACTED	Role on Project Collaborator
Organization New England BioLabs, Inc.		Department Eucaryotic Biol.
Name SHILDKRAUT, Ira	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Director of Research	D.O.B.	Role on Project Collaborator
Organization New England BioLabs, Inc.		Department Research & Devel.
Name SILVERSTEIN, Saul	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Columbia University		Department Microbiology
Name SNINSKY, John	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Senior Director of Research	D.O.B. REDACTED	Role on Project Collaborator
Organization Roche Molecular Systems, Inc.		Department PCR Research
Name SOBEL, Mark	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Chief, Molecular Pathology Section	D.O.B. REDACTED	Role on Project Collaborator
Organization National Cancer Institute		Department Pathology
Name SOMMER, Steven	Degree(s) M.D./Ph.D.	Social Security # REDACTED
Position Title Associate Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Mayo Clinic		Department Biochem, Mol.Biol.
Name SOUSSI, Thierry	Degree(s) Ph.D.	Social Security # Not applicable
Position Title Professor	D.O.B. REDACTED	Role on Project Collaborator
Organization Institute de Genetique Moleculaire		Department Molecular Genetics
Name SWERDLOW, Harold	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Research Associate	D.O.B. REDACTED	Role on Project Collaborator
Organization University of Utah		Department Human Genetics
Name VAGNER, Josef	Degree(s) Ph.D.	Social Security # REDACTED
Position Title Postdoctoral Fellow	D.O.B. REDACTED	Role on Project
Organization University of Minnesota		Department Chemistry

Name	VAGNEROVA, Lydie	Degree(s)	B.S.	Social Security #	Pending
Position Title	Research Technician	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry
Name	WANG, Guangyi	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Fellow	D.O.B.	REDACTED	Role on Project	
Organization	Purdue University			Department	Medicinal Chem.
Name	WHITE, Perrin	Degree(s)	M.D./Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Cornell University Medical College			Department	Pediatrics
Name	WILSON, Geoffrey	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Research Group Leader	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	New England Biolabs, Inc.			Department	Research
Name	WILSON, Vincent	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Associate Professor / Director	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	The Children's Hospital & Univ. of Colorado School Of Medicine			Department	Pathology
Name	WINN-DEEN, Emily	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Staff Scientist	D.O.B.	REDACTED	Role on Project	Collaborator
Organization	Applied Biosystems, Inc.			Department	Research & Devel.
Name	ZHANG, Peiming	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Research Assistant	D.O.B.	REDACTED	Role on Project	
Organization	Purdue University			Department	Medicinal Chem.

TABLE OF PROPORTIONAL EFFORT OF INVESTIAGTORS

Investigator	Project 1	Project 2	Project 3	Project 4	Project 5	Core A	Core B	Core C	Total	Other Support
A. Aggarwal				10%					10%	75%
F. Barany		15%		10%			10%	5%	40%	65%
G. Barany					10%				10%	67%
D. Bergstrom			10%						10%	50%
N. Hackett						20%			20%	50%
R. Hammer			26%						26%	35%
M. Lubin		10%					10%		20%	0%
V. Wilson	20%								20%	50%

TABLE OF CONTENTS

Project 2. Genetic Markers of Breast and Cervical Cancer

Project Leader: Dr. F. Barany

Project Co-Leader: Dr. M. Lubin

Project 3. Design and Synthesis of Nucleotide Analogues

Project Leader: Dr. D. Bergstrom

Project Co-Leader: Dr. R. Hammer

Project 4. Engineering an Improved Thermostable Ligase

Project Leader: Dr. F. Barany

Project Co-Leader: Dr. A. Aggarwal

Project 5. Design and Synthesis of DNA and PNA Arrays

Project Leader: Dr. G. Barany

Core A. Informatic Support for Cancer Detection Methods

Core Leader: Dr. N. Hackett

Core B. Instrumentation and Mutation Detection

Core Leader: Dr. F. Barany

Core Co-Leader: Dr. M. Lubin

Core C. Administrative Core

Core Leader: Dr. F. Barany

Core Co-Leader: Dr. M. Bunk

Project 5.

Design and Synthesis of DNA and PNA Arrays

Project Leader: George Barany
University of Minnesota

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. **DO NOT EXCEED THE SPACE PROVIDED.**

The goal of this program project is to develop methods for identifying multiple gene mutations in cancers. For maximum utility, these methods must be able to recognize and discriminate between dozens or hundreds of mutations.

To accomplish this, we propose to capture specific ligase detection reaction (LDR) products on a spatially addressable array, such that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be selectively captured by a "complementary zip code" on a solid support. The complementary componenets can be DNA oligonucleotides or peptide nucleotide analogues (PNA). PNA/DNA hybrids have significantly higher T_m values than DNA/DNA hybrids. Incorporation of the nucleotide analogue, 5-propynyluridine, into DNA zip code and PNA address sequences will further increase and optimize T_m values (Project 3). Unreacted LDR primer may therefore be washed away at high temperatures allowing for a higher sensitivity in detecting LDR products. A reusable, universal addressable array could be used for detecting a wide range of cancer mutations, genetic diseases and infectious agents.

Implementation of these concepts, with the ultimate goal of achieving reliable and efficient materials and procedures that can be incorporated into easy-to-use, automated, low-cost diagnostic devices, will follow these aims: (i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. Surfaces, beads, or membranes will be functionalized, and extended as needed with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates. Chemistry for linking oligomers to the solid support, and/or solid-phase assembly of oligomers, will be developed. (ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. Appropriate masking technology will expose defined regions of the solid support for attachment of pre-formed oligomers, or for chain elongation to assemble the needed oligomers. In the latter mode, segment condensation will be used when possible in order to provide efficient convergent synthesis, and because chemical "failures" will become "invisible" during the subsequent hybridization. (iii) Demonstration of scope and limitations of zip code concepts. As aims (i) and (ii) come to fruition, testing will be carried out (Core B). Design of primer and zip code structures will be facilitated by the informatics collaboration (Core A).

PERSONNEL ENGAGED ON PROJECT, INCLUDING CONSULTANTS/COLLABORATORS. *Use continuation pages as needed* to provide the required information in the format shown below on *all* individuals participating in the project.

Name	BARANY, George	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Professor	D.O.B.	REDACTED	Role on Project	Prin. Investig.
Organization	University of Minnesota			Department	Chemistry
Name	HAMMER, Robert	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Assistant Professor	D.O.B.	REDACTED	Role on Project	Co-investigator
Organization	Louisiana State University			Department	Chemistry
Name	VAGNER, Josef	Degree(s)	Ph.D.	Social Security #	REDACTED
Position Title	Postdoctoral Associate	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry
Name	VAGNEROVA, Lydie	Degree(s)	B.S.	Social Security #	pending
Position Title	Research Technician	D.O.B.	REDACTED	Role on Project	
Organization	University of Minnesota			Department	Chemistry
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	
Name		Degree(s)		Social Security #	
Position Title		D.O.B.		Role on Project	
Organization				Department	

DD

Principal Investigator/Program Director (Last, first, middle): F. BARANY, Ph.D.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 94/12/01 THROUGH 95/11/30

PERSONNEL (Applicant Organization Only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTALS
George Barany (AY)	Principal Investigator	9	5				
George Barany (SS)	Principal Investigator	3	16.7				
Josef Vagner	Post-Doc Associate	12	100				
Lydie Vagnerova	Research Technician	12	100				
PROJECT 5							
SUBTOTALS →					$55,623	$8,446	$64,069

CONSULTANT COSTS

EQUIPMENT (Itemize)

$0

SUPPLIES (Itemize by category)
Chromatography $3,000
Chemicals $5,000
Special Solvents & Reag for PNA synthesis $7,500

$15,500

TRAVEL
One trip per year for P.I. to present results $1,200

$1,200

PATIENT CARE COSTS
INPATIENT $0
OUTPATIENT $0

ALTERATIONS AND RENOVATIONS (Itemize by category)

$0

OTHER EXPENSES (Itemize by category)
See following page $5,000

$5,000

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD **$85,769**

CONSORTIUM/CONTRACTUAL COSTS
DIRECT COSTS
INDIRECT COSTS 40% Direct

TOTAL → $34,308

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page) → **$120,077**

DD

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD — PROJECT 5
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: Salary & fringe benefits Applicant organization only		$64,069	$66,632	$69,297	$72,069	$74,952
CONSULTANT COSTS		$0	$0	$0	$0	$0
EQUIPMENT		$0	$2,000	$2,000	$2,000	$2,000
SUPPLIES		$15,500	$16,120	$16,765	$17,436	$18,133
TRAVEL		$1,200	$1,248	$1,298	$1,350	$1,404
PATIENT CARE COSTS	INPATIENT	$0	$0	$0	$0	$0
	OUTPATIENT	$0	$0	$0	$0	$0
ALTERATIONS AND RENOVATIONS		$0	$0	$0	$0	$0
OTHER EXPENSES		$5,000	$5,200	$5,408	$5,624	$5,849
SUBTOTAL DIRECT COSTS		$85,769	$91,200	$94,768	$98,479	$102,338
CONSORTIUM/ CONTRACTUAL COSTS		$34,308	$35,680	$37,107	$38,591	$40,135
TOTAL DIRECT COSTS		$120,077	$126,880	$131,875	$137,070	$142,473
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 8a)->						$658,375

JUSTIFICATION (Use continuation pages if necessary):

From Budget for Initial Period: Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

For All Years: Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

From Budget for Entire Period: Identify with an asterisk (*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

For Competing Continuation Applications: Justify any significant increases or decreases in any category over the current level of support.

INITIAL BUDGET PERIOD:

General: This budget covers only the expenses in Dr. George Barany's laboratory at the University of Minnesota, and represents modest levels by comparison to other ongoing and past grants from NIH. The significant intellectual and experimental contributions to the project of Dr. Robert P. Hammer from Louisiana State University, and Dr. Francis Barany of Cornell University Medical College, are supported by separate budgets in this program project.

Personnel: The preparation, manipulation, and characterization of oligonucleotide and PNA building blocks and oligomers, as well as of a range of solid supports needed for synthesis and hybridizations, is quite labor-intensive and requires experienced and well-trained co-workers. Dr. Josef Vágner is a highly qualified peptide chemist who has been working in Dr. Barany's laboratory for over a

year on other projects. Ms. Vágnerova holds a degree in biochemistry and has recently arrived in this country to join the research group as a technician. These two individuals will be able to make an immediate impact to this Research Plan. Salaries follow University of Minnesota guidelines, and fringe benefits are calculated at 23.7% academic, 3.6% postdoctoral, and 27.5% civil service. Professors have 9-month academic appointments, and need to cover the 3-month summer salary from grants.

Equipment: Dr. Barany's laboratory has all of the major instrumentation required to carry out this research, with several new instruments acquired recently to replace and/or augment older models (listed with "Resources and Environment").

Supplies and Other Expenses: We are perpetually underfunded in these categories, and require a combination of grants to cover these costs. "Supplies" include chemical reagents, consumable supplies, and chromatography expenses. "Other Expenses" cover analytical fees (NMR, mass spectrometry, elemental analysis), instrument maintenance (service contracts on peptide synthesizers and amino acid analyzer shared with other grants), publication costs, communications, etc.

Travel: Funds are requested to allow attendance at one professional meeting per year in order to present results and learn of advances in scientific fields related to this proposal.

Consortium/Contractual Costs: The University of Minnesota negotiated (May 13, 1992) indirect cost rate is 40% of modified direct costs (total direct costs - equipment - graduate student benefits).

CONTINUATION YEARS:

Personnel: Dr. Barany's research program attracts a constant stream of postdoctoral applicants from good laboratories throughout the world, so there will be no difficulty in appointing individuals to continue the work after Dr. Vágner leaves. Similarly there is a good pool of candidates for technician positions. The "Research Plan" will require constant staffing at the level of two individuals.

Equipment: We request $2,000 per year to cover relatively small items of lab hardware.

Increases: Following NIH guidelines, the percentage recurring annual increase in all costs are calculated at 4%.

Five years of support are requested in order to allow enough time to show significant progress on the goals of the "Research Plan."

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☐ Laboratory: The Barany group currently numbers about a dozen full-time researchers, and two and a half 490 ft^2 laboratories (Kolthoff 476, 468, and 470; listed in order of length of occupancy; 4 desks per lab; common service corridor). These labs are down the hall from the faculty office. A 260 ft^2 laboratory (Kolthoff 463A, 1 desk) adjoins the office and is used for work by Dr. Barany and a laboratory technician and /or undergraduate research assistants. The group also occupies a 300 ft^2 instrumentation room (Kolthoff 472), and has a 125 ft^2 section of a shared instrumentation room (498A). All of this is in the Department of Chemistry on the Minneapolis campus of the University of Minnesota.

☐ Clinical:

☐ Animal:

☐ Computer: Three Macintosh personal computers for word processing and graphics

☐ Office: 140 ft^2 (Kolthoff 461)

☐ Other ( ):

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

MilliGen/Biosearch 9050 and 9600 Peptide Synthesizers (acquired 1990); Beckman System 6300 High Performance Amino Acid Analyzer (acquired 1989); Beckman-Altex analytical gradient HPLC apparatus (acquired 1981) with variable wavelength UV detector and Hewlett-Packard recording integrator; Waters Delta-Prep 3000 HPLC (acquired 1988) apparatus with UV detector, integrator, and automatic sample injector; Beckman P/ACE 2100 capillary zone electrophoresis system (acquired 1991); MPLC set-up; UV-visible spectrophotometer; photolysis equipment; fraction collectors; Labconco lyophilizer; basic organic synthesis set-up; excellent hoods in all of the laboratories.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Buildings of the Department of Chemistry contain major instrumental facilities for routine and high-field ^{1}H and ^{13}C-NMR, IR, mass spectrometry (including FABMS), and X-ray diffraction which are extensively used in this research program. Machine, electronics, and glassblowing shops are on-site, as is a research stockroom. The Department offers quarter-time secretarial support to the faculty, and Dr. Barany occasionally gets additional secretarial help paid for by research grants. An additional resource for this research is the Microchemical Facility of the University of Minnesota Institute of Human Genetics on the Minneapolis campus, which includes state-of-the-art equipment for amino acid analysis, peptide sequencing and synthesis, oligonucleotide synthesis, and other procedures.

A. SPECIFIC AIMS:

This Research Plan seeks to develop and optimize new tools that will be essential components to the integrated approach to cancer, genetic, and infectious disease detection described in the overall program project application. A novel polymerase chain reaction/ligase detection reaction (PCR/LDR) method for discriminating normal, carrier, and disease individuals has been described, and a high-sensitivity PCR/restriction endonuclease/LDR (PCR/RE/LDR) method for detection of rare cancer mutations is under development (Projects 1 and 2). These technologies, in their present form or as improved by applications of "convertide" nucleotide analogue bases (Project 3) and/or engineered thermostable ligase (Project 4), will be carried out in multiplex formats to simultaneously identify many mutations. LDR products, derived from one fluorescent primer and an adjacent primer with extra nucleotides or hexaethylene oxide "tails", are currently separated by size on an automated DNA sequencer, or by capillary electrophoresis. Use of different fluorescent groups allows a second dimension of mutation discrimination.

Herein, we propose new solid-phase approaches for simultaneous detection of multiplex LDR products. The general idea is that specific products will be captured on a spatially addressable array, so that the position of a signal identifies a mutation. Each LDR product will have a "zip code" tail, which will be captured selectively by a "complementary zip code" on the solid support. The supported (complementary) components can be modified DNA oligonucleotides or peptide nucleotide analogues (PNA), designed so that the resultant zip code hybrids have a significantly higher T_m than DNA/DNA hybrids. Unreacted primers may be washed away at high temperatures, allowing for detection of the LDR product. Multiple reuse of a universal "complementary zip code" array is envisaged to allow detection of a wide range of cancers and genetic diseases.

Implementation of these concepts, with the ultimate goal of achieving reliable and efficient materials and procedures that can be incorporated into easy-to-use, automated, low-cost diagnostic devices, will follow these aims:

(i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. Both commercially available and experimental materials will be screened. Surfaces, beads, or membranes will be functionalized, and extended as needed with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates. Chemistry for linking oligomers to the solid support, and/or solid-phase assembly of oligomers, will be developed.

(ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. Appropriate masking technology will expose defined regions of the solid support for attachment of pre-formed oligomers, or for chain elongation to assemble the needed oligomers. In the latter mode, segment condensation will be used when possible in order to provide efficient convergent synthesis, and because chemical "failures" will become "invisible" during the subsequent hybridization.

(iii) Demonstration of scope and limitations of zip code concepts. As aims (i) and (ii) come to fruition, testing will be carried out (Core B). Design of primer and zip code structures will be facilitated by the informatics collaboration (Core A).

B. BACKGROUND AND SIGNIFICANCE

The cancer-detection technology of this program project application relies in good part on the capability to prepare by rapid, accurate chemical methods a multitude of oligonucleotide and related structures of defined sequence in the 20 to 50-base size range. The revolutionary solid-phase approach, introduced by Merrifield in the 1960's for peptides, points the way and provides ample experiences and precedents [1-6]. In solid-phase methodology that has been refined substantially and is readily automated, suitably protected amino acid building blocks are added in order (C to N) to a growing chain which is attached covalently through the C-terminus to a polymeric support. The principal commercially available

chemistries are referred to as "Boc" and "Fmoc", abbreviations for the names of the key *temporary* protecting groups. Often, a *linker* or *handle* is used to mediate the initial anchoring. Reactions are driven to completion by the use of excess reagents, which are removed by simple filtration and washing steps; the chemistry can also be carried out in columns in a continuous-flow mode. Upon completion of chain assembly, protecting groups are cleaved and the peptide is released into solution for further purification and characterization. An important aspect for successful results is the choice of the polymeric support. For many years, most work was carried out on 1% cross-linked microporous polystyrene resins (beaded), or on polyacrylamides (these latter could be embedded within an inorganic matrix, e.g., kieselguhr, or a rigid macroporous polystyrene, e.g., Polyhipe) [6-12]. Within the past few years, several additional materials with interesting physico-chemical properties have become available from several academic laboratories and commercial sources. These include membranes [13, 14], cotton and other appropriate carbohydrates [15-18], controlled-pore silica glass [19], and linear polystyrene grafted onto Kel-F [20]. A particularly interesting concept involves the use of polyethylene glycol-polystyrene supports (PEG-PS or Tentagel), which are compatible with both batchwise and continuous-flow reactors, and may facilitate difficult chemistries in peptide synthesis [21-23]. Other recent trends from the peptide field which are relevant to the goals of this proposal involve synthesis on polymeric surfaces, and the simultaneous preparation of multiple structures. In these procedures, due to Geysen, Frank, and research groups at Affymax, Arris, and Millipore, among others, relatively short peptides are built up on appropriately modified polyethylene pins, cellulose or polypropylene membranes, or glass surfaces, in a way that the bound final structures (purities in the 60 to 90% range) are tested directly by ELISA or other biological testing methods [15, 24-27]. The active structures are then deduced from their physical position, i.e., *spatial address* on the array. In an alternative combinatorial *library* approach developed at Selectide [28], millions of peptides are generated simultaneously by successive cycles of randomization/remixing of beaded supports. Intrinsic to the design of these experiments, each individual bead contains only a single peptide, so that those beads giving a positive interaction with a receptor can be picked out and subjected to analytical procedures that give the structure on the bead.

Solid-phase oligonucleotide synthesis has come to the fore in the past eight years with the development of reliable high-efficiency phosphoramidite [29] or H-phosphonate chemistry [30, 31] for linking protected nucleoside building blocks. Synthesis (3' to 5') is usually supported on controlled-pore glass, although other materials can also serve. The current automated methodology can routinely furnish oligonucleotides of length > 50 residues in overall purity > 90% directly upon release from the support. The methodology can also be adapted to incorporate unusual nucleotide bases, as well as modifications in the phosphodiester backbone (e.g., non-bridging thio or dithio substitution) and end-group labels (e.g., fluorescent dyes, biotin) [32]. As with peptides, relatively short oligonucleotides can be synthesized in spatially addressable arrays on glass surfaces [33-37]. Alternatively, a variety of procedures have been described for site-specific attachment of pre-synthesized oligonucleotide probes to nylon membranes or inside polyacrylamide gels [38-40]. Such arrays have been applied for DNA hybridization reactions, with applications to DNA sequencing or detection of biotinylated PCR-amplified products. These earlier studies provide useful precedents to some of the goals of this proposal.

Within the last few years, a group from Denmark [41-44] has introduced novel peptide nucleotide analogue (PNA) oligomers which mimic closely the spatial arrangement of the oligonucleotide backbone, but use nonchiral (2-aminoethyl)glycine units to replace the sugar phosphodiester (Figure 1). Additional innovations for PNA chemistry are under development at Millipore, in close consultation and collaboration with us [45]. These improvements include complete protection schemes for all the "bases" in concert with Boc, Fmoc, or alternative chemistries, optimized coupling (note: since racemization is not an issue, strong activation methods can be applied) and capping protocols, and efficient sequencing procedures. PNA and single-stranded DNA join to form anti-parallel heteroduplexes that exhibit Watson-Crick specificity and (particularly under low-salt conditions) tighter binding (higher T_m) than the corresponding double-stranded DNA [44]. As is discussed later, these properties dovetail extremely well with some of the requirements for the multiplex cancer detection protocol proposed in this program project application.

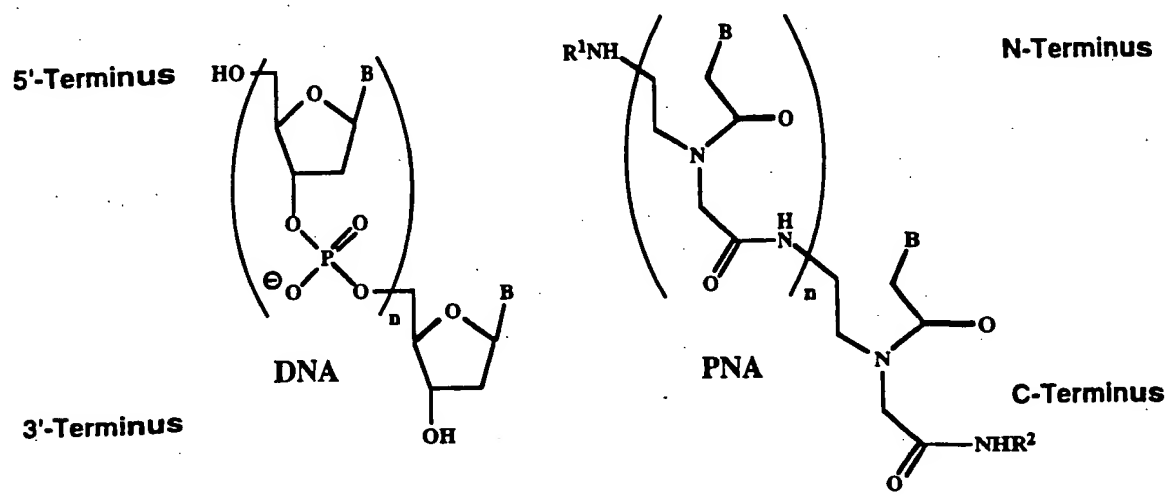


Fig. 1. Structural similarity of DNA and PNA.

C. PRELIMINARY STUDIES

The previous section of this proposal gave a brief overview of the current status of solid-phase methodologies, with an emphasis on general aspects which are relevant to the objectives of the research program. The following paragraphs provide brief descriptions of recent experimental advances from our laboratories which place us at the cutting edge of methodological developments.

We have invented several procedures for grafting both homo- and heterobifunctional polyethylene glycol (PEG) derivatives of defined molecular weight onto amino-functionalized polystyrene (PS); the resultant microporous beaded PEG-PS supports are now commercially available through Millipore and have numerous advantageous properties with respect to polystyrene [22, 23]. Our extensive experience in this field will be needed to introduce PEG as a "spacer" separating oligonucleotide or PNA molecules from functionalized surfaces. A useful start in this regard was reported recently by our collaborators Dr. Dereck Hudson and Dr. Ronald Cook [27] who in turn derivatized polyethylene plates, modified the resultant surfaces with PEG, and coupled carboxymethyl dextran to impart further hydrophilicity and serve as a starting point for peptide synthesis. (Please see letters of collaboration in overview section of program project).

PEG-PS has proven to be an ideal support for peptide library studies by the Selectide process [28]. PEG-PS is compatible with the organic reagents and solvents for efficient synthesis throughout the beads, and also has sufficient hydrophilic character to allow biological testing in aqueous milieus. Based on the realization that biological interactions occur only at the surface of beads, we have devised methodology for differentiation of "surface" and "interior" areas. PEG-PS is loaded with Boc-Trp-Gly, following which chymotryptic digestion "shaves" selectively only substrates at the surface which are accessible to the macromolecular enzyme. The exposed glycine is the starting point for orthogonal peptide synthesis using Fmoc chemistry, so that each bead is charged with a "screening" peptide at the surface, representing <1% of the total content but responsible for the entire spectrum of biological interaction. In concert, Boc chemistry establishes a sequenceable "coding" peptide confined to the interior, representing the vast majority of material on the bead but restricted from biological interaction [46]. This "shaving" concept has implications beyond the application to encoded combinatorial libraries just described; in the context of the present Research Plan, it can be used to ensure that oligopolymers synthesized on surfaces will be able to hybridize to oligonucleotides.

Our laboratories have also pioneered the development of novel linkers and handles for peptide synthesis [47, 48]; the extension to DNA and PNA is expected to be straightforward. Of particular interest, we have developed tris(alkoxy)benzyl amide (PAL) [49] and ester (HAL) [50] linkages, which upon cleavage with acid provide respectively C-terminal peptide amides, and *protected* peptide acids that can be

used as building blocks for so-called *segment condensation* approaches. We have noticed that the stabilized carbonium ion generated in acid from cleavage of PAL or HAL linkages can be intercepted by tryptophanyl-peptides. While this reaction is a nuisance for peptide synthesis and preventable (in part) by use of appropriate scavengers, we envisage herein a positive application to chemically "capture" oligo-Trp-end-labelled DNA and PNA molecules by HAL-modified surfaces (see Fig. 7 in Experimental Designs and Methods).

D. EXPERIMENTAL DESIGN AND METHODS

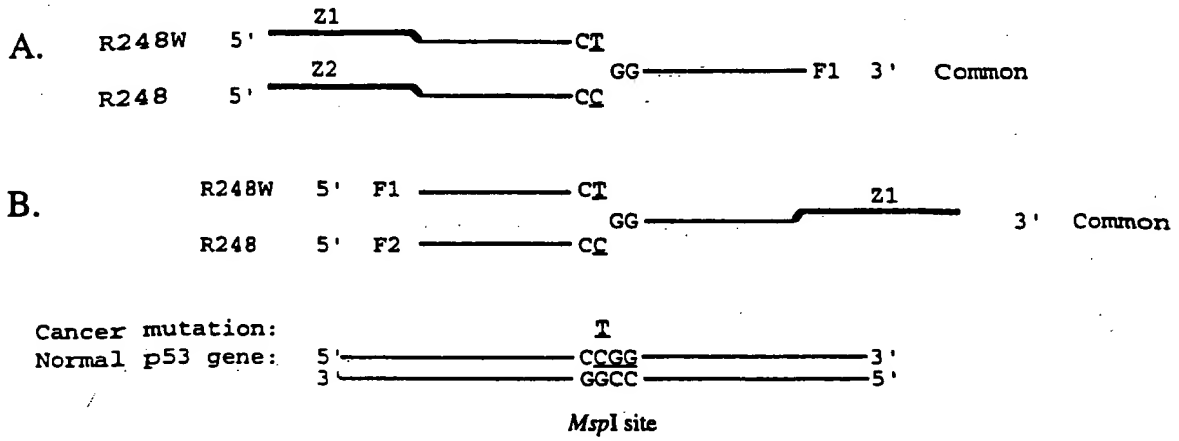
(i) Overview

(a) Array technology. This Reseach Plan describes a systematic approach to the design and synthesis of oligonucleotide or PNA arrays to achieve accurate detection and quantification of cancer mutations. Several groups have attempted to make oligonucleotide arrays with various degrees of success [33, 37- 40]. These approaches may be divided into three categories: (i) Synthesis of oligonucleotides by standard methods and their attachment one at a time in a spatial array [38-40] (ii) Photolithographic masking and photochemical deprotection on a silicon chip, to allow for synthesis of short oligonucleotides [37], and (iii) Physical masking to allow for synthesis of short oligonucleotides by addition of single bases at the unmasked areas [33, 36]. Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, severe limitations have been noted in using these arrays for detecting specific DNA sequences by hybridizations. Arrays containing longer oligonucleotides can currently be synthesized only by attaching one address at a time, and thus are limited in potential size. (Current methods for attaching an oligonucleotide take about 1 hour, thus an array of 1,000 addresses would require over 40 days of around-the-clock work to prepare.) The "reverse dot blot" approach is capable of distinguishing single base differences in homozygous or heterozygous individuals, as well as the presence of a *ras* mutation diluted 20-fold by normal DNA [40]. However, hybridization methods require careful attention to temperature and salt conditions, and cannot achieve the high sensitivity of the cancer detection methods described in this proposal. Arrays containing large numbers of short oligonucleotides have performed significantly better on the computer than in practice. Syntheses on membranes or silicon chips are plagued by less than 100% efficiency, effectively limiting the size of these oligonucleotides to 8- to 10-mers. Imperfect hybridizations generate significant background signals, which severely hamper use of these arrays for DNA sequencing [36].

(b) Zip code concept. This proposal introduces a novel approach to oligonucleotide arrays which should obviate the above problems. One significant difference between our approach and literature array methods is that we use the array as a means to capture the *correctly generated* product. While others try to distinguish closely related sequences by subtle differences in melting temperatures during hybridization, we have already achieved the required exquisite specificity due to the discriminating actions of thermostable ligase in solution. Thus, our arrays can be designed to contain sequences which are ***very different*** from each other. Our array may be likened to 1,000 different antibodies that bind 1,000 different antigens with tight binding constants and no cross-reactivity. These arrays are completely universal, so that a single design may be used in detection of infectious and genetic diseases, or cancers. Best of all, the arrays will be highly stable and reusable.

The 1,000 different "antigens" are unique 24-mer "zip code" sequences linked covalently to the approximately 20- to 25-mer target-specific sequence of an LDR primer. A "zip code" sequence does not have any homology to either the target sequence or to other sequences on the genome. This zip code tail is then captured by its "antibody", a sequence complementary to the zip code on the addressable solid support array. The concept is shown in two possible formats for detection of the p53 R248 mutation (Fig. 2). At the top of the diagram shows two alternative formats for primer design to identify the presence of a germ line mutation in codon 248 of the p53 tumor suppression gene. The wild type sequence codes for arginine (R248) while the cancer mutation codes for tryptophan (R248W). The lower part of the diagram is a schematic for zip code capture. In the first format (A), the discriminating primers contain the allelic specific base (T for mutant and C for wild type) on the 3' end and 24-mer zip codes Z1 and Z2 on their 5' ends respectively. A common downstream primer contains a fluorescent group F1 at its 3' end. In the presence

presence of appropriate target DNA (wild type DNA is shown), the correct ligation products form. After hybridization of the zip code primers to their complementary sequences on the addressable array, unreacted fluorescent primers will be washed away. Mutant and wild type signal may be quantified using a FluorImager, and distinguished by their position on the array. In an alternative format (B), the discriminating oligonucleotides contain two different fluorescent groups F1 and F2, while the common oligonucleotide contains the zip code Z1. In this format, mutant and wild type signal are distinguished by the differences in fluorescence between F1 and F2 (see legend of Fig. 2 for more details).



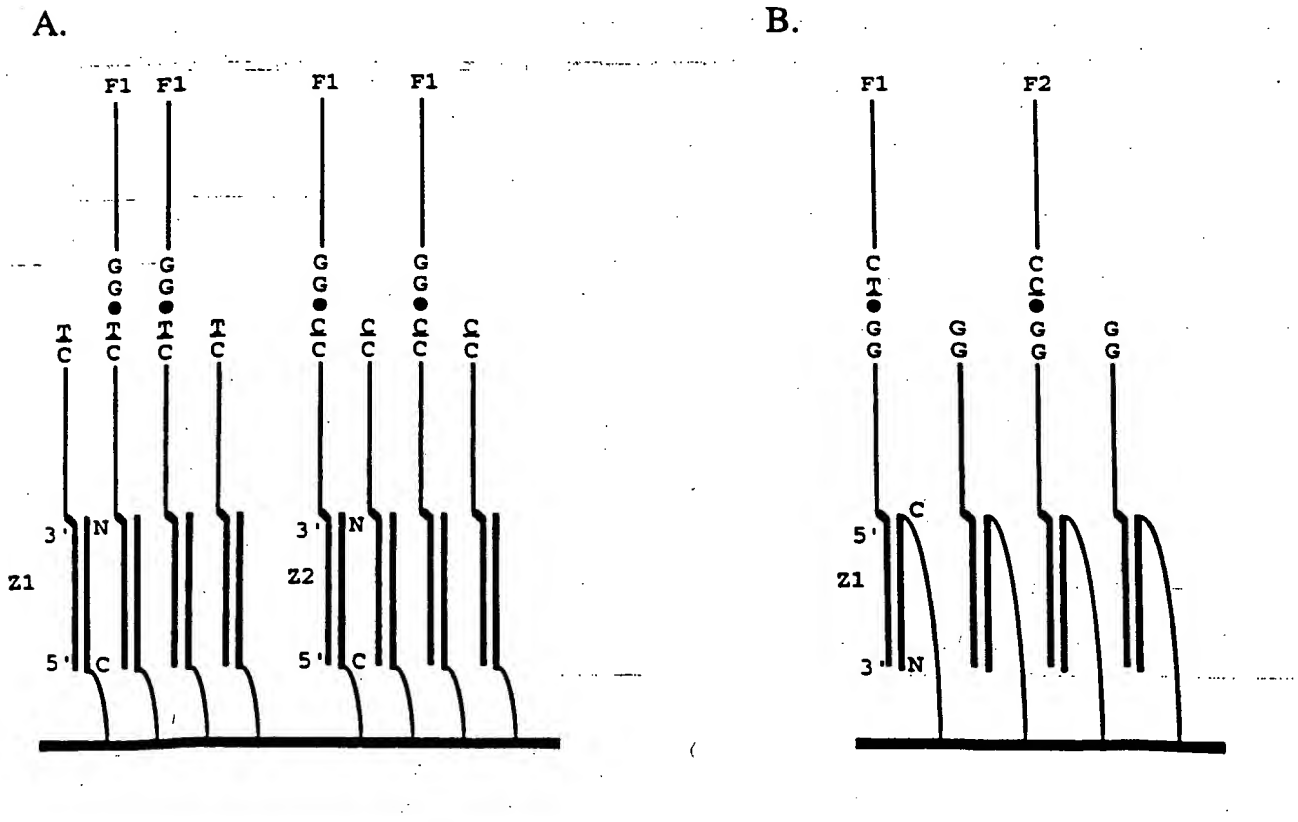


Fig. 2. Two alternative formats for zip code capture (see following page for legend).

Two alternative formats for zip code capture (legend for previous page. The top portion of the diagram shows two alternative formats for primer design to identify the presence of a germ line mutation in codon 248 of the p53 tumor suppressor gene. The wild type sequence codes for arginine (R248), while the cancer mutation codes for tryptophan (R248W). The bottom part of the diagram is a schematic diagram of zip code capture. The thick horizontal line depicts the membrane or solid surface containing the addressable array. The thin curved lines indicate a flexible linker arm. The thicker lines indicate a PNA sequence, attached to the solid surface in the C to N direction. For illustrative purposes, the PNA oligonucleotides are drawn vertically, making the linker arm in section B appear "stretched". Since the arm is flexible, the oligonucleotide will be able to hybridize 5' to C and 3' to N in each case, as dictated by base pair complementarity. A similar orientation of DNA/PNA hybridization would be allowed if the PNA were attached to the membrane at the N-terminus. Similar considerations apply when the complementary zip code on the support is a DNA oligonucleotide rather than PNA. (A) Two LDR primers are designed to discriminate wild type and mutant p53 by containing the discriminating base C or T at the 3' end. In the presence of the correct target DNA and *Tth* ligase, the discriminating primer is covalently attached to a common downstream oligonucleotide. The downstream oligonucleotide is fluorescently labeled. The discriminating oligonucleotides are distinguished by the presence of a unique "zip code" sequences, Z1 and Z2, at each of their 5' ends. A black dot indicates that target dependent ligation has taken place. After ligation, all zip code primers may be captured by their complementary "zip code" sequences at unique addresses on the array. Both ligated and unreacted primers are captured by the PNA array. Unreacted fluorescently labeled common primers and target DNA are then washed away at a high temperature (approximately 65°C to 80°C) and low salt. Mutant signal is distinguished by detection of fluorescent signal at the Z1 position, while wild type signal appears at the Z2 position. Heterozygosity is indicated by equal signals at both Z1 and Z2. The signals may be quantified using a Molecular Dynamics FluorImager. This format uses a unique address for each allele, and may be preferred for achieving very accurate detection of low levels of signal (30 to 100 attomoles of LDR product). (B) In this format, the discriminating oligonucleotides are distinguished by having different fluorescent groups, F1 and F2, on their 5' end. Either oligonucleotide may be ligated to a common downstream oligonucleotide containing a zip code sequence Z1 on its 3' end. In this format, both wild type and mutant LDR products are captured at the same address on the array, and are distinguished by their different fluorescence. This format allows for a more efficient use of the array and may be preferred when trying to detect hundreds of potential germline mutations.

(c) Design and synthesis of arrays. This Research Plan will explore variations of two general approaches for synthesizing arrays. In the first approach, we will prepare full-length 24-mer DNA oligonucleotides or PNA oligomers, which are subsequently linked covalently to a solid support or membrane. Alternatively, the deprotected DNA or PNA may remain linked to the bead, and the entire bead glued to a solid support. In the second approach, 36 specially designed PNA tetramers will be synthesized. These tetramers will be added to specific rows or columns on a solid support or membrane surface. The resulting "checkerboard" pattern will generate unique addressable arrays of PNA 24-mers.

We will initially explore glass and derivatized membrane supports to test their sensitivities and capacities as array surfaces. Pilot experiments will involve synthesis of five zip code PNA oligomers or oligonucleotides (sequences listed in Table 2, later). These oligomers will be covalently linked to the test surfaces. Fluorescently labeled complementary DNA zip code sequences will be synthesized in Core B, and used for testing arrays produced in this project. Note that for the initial studies, we do not require the longer conjugates that combine the LDR primer with the zip code (see Fig. 1).

What properties are desired in an array? The most important factor is good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. The current commercially available fluorescent imager can detect a signal as low as 2 attomoles per 50μ square pixel. Thus, a reasonable size address or "spot" on an array would be about 4 x 4 pixels, or 200μ square. The limit of detection for such an address would be about 32 attomoles per "spot", which is comparable to the 100 attomole detection limit using a DNA sequencing machine. The capacity of oligonucleotide which can be loaded per 200μ square will give an indication of the potential signal to noise ratio. A loading of 20 fmoles would give a signal to noise ratio of 625 to 1, while 200 fmoles would allow for a superb signal to noise ratio of 6,250 to 1. Loadings in excess of 200 fmoles will be unnecessary, since most LDR reactions use only 200 fmoles of each primer. The oligonucleotide or PNA oligomer should be on a flexible "linker arm" and on the "outside" or "surface" of the solid support for easier hybridizations. The support should be non-fluorescent, and should not interfere with hybridization nor give a high background signal due to nonspecific binding. In a mode where bead(s) are attached (typical size 50-200μ), neither beads nor "glue" should give a high background signal due to nonspecific binding or intrinsic fluorescence.

This proposal also introduces a novel approach for the design and synthesis of a universal PNA oligonucleotide array with 1,296 addresses. We envision each address to be about 200μ with an equal size

space in between addresses. Feasibility will be assessed with a 25 address array that is about 2 mm square; the full-sized array would be about 1.4 cm square. Preparation of such arrays (pilot and full-sized) will be carried out as a joint project with our industrial collaborators Dr. James Coull and his team at Millipore, and Dr. Ronald Cook who heads Siris Labs. (Please see letters of collaboration in overview section). The required preliminary synthesis in the academic laboratories will use a Biorad dot blot apparatus which contains individual microtiter wells sandwiched around a membrane. This allows for addition and filtration of chemicals in each well.

(ii) Design and optimization of zip codes and addresses

(a) General considerations. The principle of using zip codes has been explained earlier. The complementary zip codes (addresses) on the solid supports can be either DNA or PNA. *Both* will be tested. However, we expect that PNA-based capture of zip codes may have advantages over DNA-based capture because PNA/DNA duplexes are much stronger than DNA/DNA duplexes, by about 1°C/base-pair [44]. Thus, for a 24-mer DNA/DNA duplex with T_m = 72°C, the corresponding duplex with one PNA strand would have a "predicted" T_m = 96°C (the actual melting point might be slightly lower as the above "rule of thumb" is less accurate as melting points get over 80°C). Additionally, the melting difference between DNA/DNA and PNA/DNA becomes even more striking at low salt.

(b) Enhancement of the hybridization affinity of zip code/address duplexes. The melting temperature of DNA/DNA duplexes can be estimated as $[4n(G•C) + 2m(A•T)]$°C. If possible, we would like to narrow the T_m difference between zip code duplexes resulting from differences in G•C/A•T content, and in this way further optimize zip code capture. Froehler has shown that use of 5-propynyl-dU in place of thymine increases the T_m of DNA duplexes an average of 1.7°C per substitution [51]. We suggest that the same substitution in the zip code capture scheme would lower the T_m difference between zip code/address duplexes, and raise the T_m for all of the zip code/address duplexes. Phosphoramidite derivatives of 5-propynyl-dU (Fig. 3) will be prepared according to Froehler [51]. The 5-propynyluracil PNA monomer with Fmoc amino protection will be made (Fig. 4) following the published synthesis of PNA monomers [41, 42], replacing thymine with 5-iodouracil and using Pd(0) coupling of the alkylated 5-iodouracil and propyne. These monomers will be incorporated into synthetic DNA and PNA strands, respectively, and evaluated as described later.

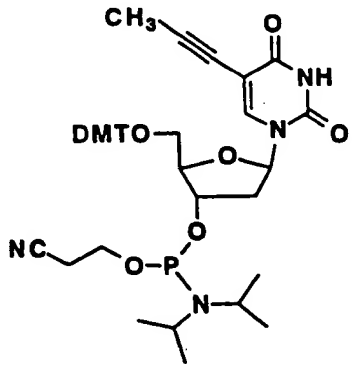
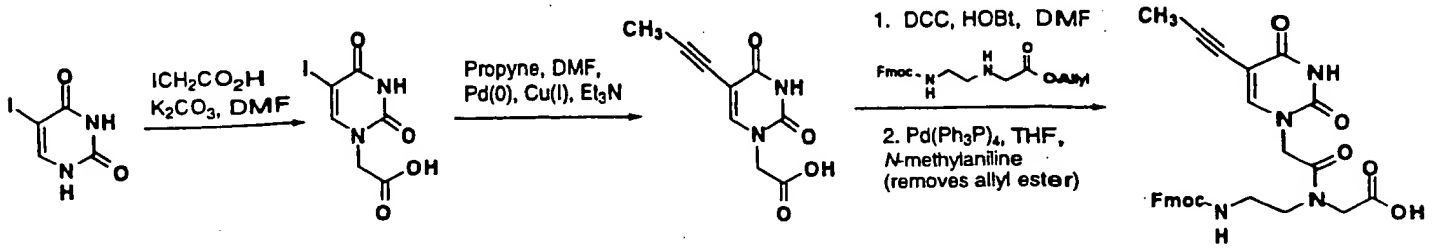


Fig. 3. Structure of nucleoside analogue 5-propynyl-dU.



The Boc-protected derivative could be made by a similar route.

Fig. 4. Synthesis of Fmoc-protected 5-propynyl-uridine PNA monomer.

(c) Zip code sequences designed from tetramer building blocks. Of the 256 (4^4) possible ways in which four bases can be arranged as tetramers, we have selected 36 that have unique sequences (Fig. 5). Each of the chosen tetramers differs from all the others by at least two bases, and no two dimers are complementary to each other. Furthermore, tetramers that would result in self-pairing or hairpin formation of the addresses have been eliminated (see legend to Fig. 5 for further details of the design process).

The final tetramers are listed in Table 1, and have been numbered arbitrarily from 1 to 36. Our premise is to use this unique set of tetramers as design modules for the required 24-mer zip code and 24-mer address sequences. The structures can be assembled by stepwise (one base at a time) or convergent (tetramer building blocks) synthetic strategies. Note that the numbering scheme for tetramers allows us to abbreviate each zip code as a string of six numbers (e.g., second column of Table 2, in following section).

(d) Initial zip code test sequences. The concept of zip code 24-mers designed from a unique set of 36 tetramers (Table 1) allows a huge number of possible structures, $36^6 = 2,176,782,336$. We have chosen five structures (Table 2) that have nearly equal G + C content as targets for the graded set of studies that are needed to establish the proposed methodology.

(e) Solution studies of zip code annealing. Our eventual goal is to exploit zip code hybridization to direct fluorescently labelled LDR products towards specific addresses on a solid support. However, first we wish to validate duplex formation in solution. Test sequences (Table 2) will be used. The T_m of each duplex will be measured by recording the A_{260} of the oligonucleotide solutions (~ 5μM concentration of each single strand) versus temperature.

Synthetic probes (normal and complementary directions) for the aforementioned studies will be prepared as either DNA or PNA, with either all thymine or all 5-propynyl-uracil. Where syntheses are straightforward, they will be performed by Core B, but where methodology is still under development, syntheses will be performed in the laboratories of program project chemists or industrial collaborators. These syntheses will generate for each sequence a total of eight oligomers, which can be combined in 16 ways that form duplexes.

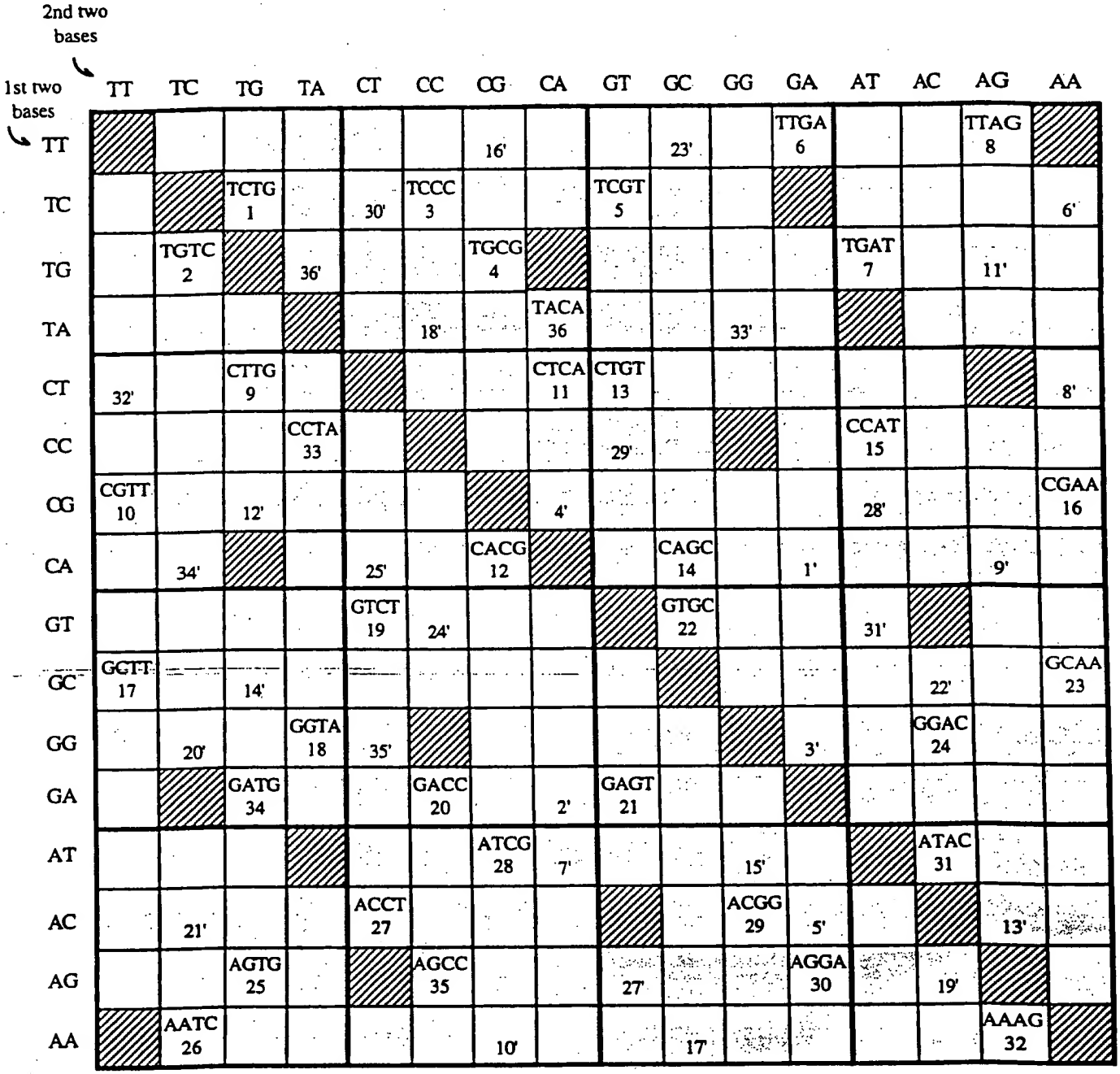


Fig. 5. Design of 36 tetramers which differ from each other by at least 2 bases. Checkerboard pattern shows all 256 possible tetramers. A given square represents the first two bases on the left followed by the two bases on the top of the checkerboard. Each tetramer must differ from each other by at least two bases, and should be non-complementary. The tetramers are shown in the white boxes, while their complements are listed as (number)'. Thus, the complementary sequences GACC (20) and GGTC (20') are mutually exclusive in this scheme. In addition, tetramers must be non-palindromic, e.g., TCGA (darker diagonal line boxes), and non-repetitive, e.g., CACA (darker diagonal line boxes from upper left to lower right). All other sequences which differ from the 36 tetramers by only 1 base are shaded in light gray. Four potential tetramers were not chosen as they are either all A•T or G•C bases. In addition, thymine can be replaced by 5-propynyl uridine when used within DNA or PNA address sequences as well as in the DNA zip code sequences. This would increase the T_m of an A•T base pair by ~1.7°C. Thus, T_m values of individual tetramers should be approximately 15.1°C to 15.7°C. T_m values for the full length 24-mers should be 95°C or higher.

Table 1. List of tetramer PNA sequences and complementary DNA sequences, which differ from each other by at least 2 bases.

Number	Sequence (N-C)	Complement (5'–3')	G + C
1.	TCTG	CAGA	2
2.	TGTC	GACA	2
3.	TCCC	GGGA	3
4.	TGCG	CGCA	3
5.	TCGT	ACGA	2
6.	TTGA	TCAA	1
7.	TGAT	ATCA	1
8.	TTAG	CTAA	1
9.	CTTG	CAAG	2
10.	CGTT	AACG	2
11.	CTCA	TGAG	2
12.	CACG	CGTG	3
13.	CTGT	ACAG	2
14.	CAGC	GCTG	3
15.	CCAT	ATGG	2
16.	CGAA	TTCG	2
17.	GCTT	AAGC	2
18.	GGTA	TACC	2
19.	GTCT	AGAC	2
20.	GACC	GGTC	3
21.	GAGT	ACTC	2
22.	GTGC	GCAC	3
23.	GCAA	TTGC	2
24.	GGAC	GTCC	3
25.	AGTG	CACT	2
26.	AATC	GATT	1
27.	ACCT	AGGT	2
28.	ATCG	CGAT	2
29.	ACGG	CCGT	3
30.	AGGA	TCCT	2
31.	ATAC	GTAT	1
32.	AAAG	CTTT	1
33.	CCTA	TAGG	2
34.	GATG	CATC	2
35.	AGCC	GGCT	3
36.	TACA	TGTA	1

(iii) Solid support materials for array technology

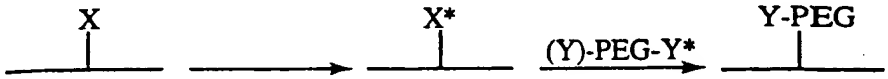
Earlier sections of this proposal have reviewed the ideal requirements for array support materials, in the context of options from the literature and our own extensive experiences. The solid supports must be charged with DNA oligonucleotides or PNA oligomers; this is achieved either by attachment of pre-synthesized probes, or by direct assembly and side-chain deprotection (without release of the oligomer) onto the support. Further, the support environment needs to be such as to allow efficient hybridization. Towards this end, three factors may be identified: (i) sufficient hydrophilic character of support material (e.g., PEG or carbohydrate moieties); (ii) flexible linker arms (e.g., hexaethylene oxide or longer PEG chains) separating the probe from the support backbone; (iii) "shaving" procedures which allow probe immobilization or probe synthesis to occur only in the most accessible "surface" areas of the support. It should be kept in mind that numerous ostensibly "flat surfaces" are quite thick at the molecular level. Lastly, it is important that the support material not provide significant background signal due to non-specific binding or intrinsic fluorescence.

Table 2. List of initial PNA zip code and complementary DNA oligonucleotides.

Polymer	Zip code	Sequence			G+C
PNA	16-3-34-2-9-1	NH_2- 16 3 34 2 9 1 CGAA-TCCC-GATG-TGTC-CTTG-TCTG	-COOH		13
DNA	1-9-2-34-3-16(c)	5'- CAGA-CAAG-GACA-CATC-GGGA-TTCG	-3'		13
PNA	7-3-11-2-18-1	NH_2- 7 3 11 2 18 1 TGAT-TCCC-CTCA-TGTC-GGTA-TCTG	-COOH		12
DNA	1-18-2-11-3-7(c)	5'- CAGA-TACC-GACA-TGAG-GGGA-ATCA	-3'		12
PNA	20-3-14-2-7-1	NH_2- 20 3 14 2 7 1 GACC-TCCC-CAGC-TGTC-TGAT-TCTG	-COOH		14
DNA	1-7-2-14-3-20(c)	5'- CAGA-ATCA-GACA-GCTG-GGGA-GGTC	-3'		14
PNA	29-3-23-2-12-1	NH_2- 29 3 23 2 12 1 ACGG-TCCC-GCAA-TGTC-CACG-TCTG	-COOH		15
DNA	1-12-2-23-3-29(c)	5'- CAGA-CGTG-GACA-TTGC-GGGA-CCGT	-3'		15
PNA	13-35-27-33-2-7	NH_2- 13 35 27 33 2 7 CTGT-AGCC-ACCT-CCTA-TGTC-TGAT	-COOH		12
DNA	7-2-33-27-35-13(c)	5'- ATCA-GACA-TAGG-AGGT-GGCT-ACAG	-3'		12

A variety of materials, which include suitably modified glass, plastic, or cellulose surfaces, PEG-PS beads, or a variety of membranes, will be examined in the context of the needs summarized above. These materials will be obtained from commercial sources or from our industrial collaborators (Dr. James Coull at Millipore, Dr. Ronald Cook at Siris), or else will be prepared in our laboratories by following literature precedents. Depending on the material, surface functional groups (i.e., hydroxyl, carboxyl, amino) may be present from the outset (perhaps as part of the coating polymer), or will require a separate procedure (e.g., plasma amination, chromic acid oxidation, treatment with a side-chain functionalized alkyltrichlorosilane) for introduction of the functional group. Hydroxyl groups become incorporated into stable carbamate (urethane) linkages by several methods. Amino functions can be acylated directly, whereas carboxyl groups are activated, e.g., with N,N'-carbonyldiimidazole or water-soluble carbodiimides, and reacted with an amino-functionalized compound (Fig. 6). Unreacted amino groups will be blocked by acetylation or succinylation, to ensure a neutral or negatively charged environment that "repels" excess unhybridized DNA. Loading levels will be determined by standard analytical methods [47].

Often, it will be desirable to introduce a PEG spacer with complementary functionalization, prior to attachment of the starting linker for DNA or PNA synthesis. The methodology to do so is in hand [22, 23, 52] and will be pursued alongside with control experiments on the same materials lacking PEG. Similarly, dextran layers can be introduced as needed by precedented chemistries [27, 53]. Finally, enzymatic "shaving" is carried out readily by our recently developed procedure using chymotrypsin to cleave a short substrate that is distributed uniformly throughout a bead or on a derivatized surface. In our studies on peptide/receptor (antibody or binding protein) interactions, we have shown that shaving protocols expose a relatively small portion (approximately 1 to 5%) of the total functional groups, yet they reach all receptor-accessible sites [46]. We plan to establish whether the same site selectivity can be achieved for hybridization reactions, and compare the results to controls run on "unshaven" materials.



X,Y = –OH, $-CO_2H$, $-NH_2$
(Y) = W–NH–

X*, Y* = $-O(C=O)Z$, $-O(C=S)Z$, $-CO_2H$, $-(C=O)Z$, $-NH_2$, $-N=C=O$

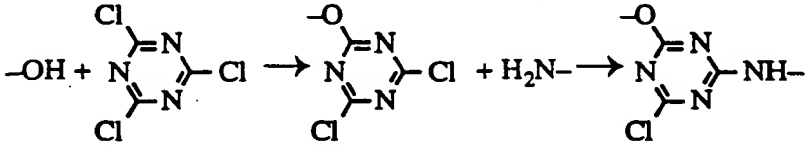
W = protecting group, e.g. Boc, Fmoc
Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp), hydroxysuccinimide (OSu), pentafluorophenol (OPfp)
PEG = oligo or poly(ethylene glycol), backbone $(CH_2CH_2O)_n$ n = 6 to 200 (can also be grown by anionic polymerization with )
WSC = water soluble carbodiimide

Functional group transformations/activation (as needed), X → X*, Y → Y*

$-OH \longrightarrow -O(CH_2)_nCO_2H$ n = 1, 2
$-OH \longrightarrow -O(C=O)NHCH_2CO_2H$
$-OH \longrightarrow -O(C=O)CH_2NH_2$
$-OH \longrightarrow -O(C=O)Im$
$-OH \longrightarrow -O(C=S)SCH_2(C=O)NH_2$
$-CO_2H \longrightarrow -(C=O)NH(CH_2)_nNH_2$ n = 2,6
$-CO_2H \longrightarrow -(C=O)Z$
$-NH_2 \longrightarrow -NH(C=O)(CH_2)_nCO_2H$ n = 2, 3

Covalent linkage, X* + Y*

$-CO_2H + H_2N- + WSC + HOSu \longrightarrow -(C=O)NH-$
$-OH + Im(C=O)Im + H_2N- \longrightarrow -O(C=O)NH-$



$-OH + O=C=N- \longrightarrow -O(C=O)NH-$
$-O(C=S)SCH_2(C=O)NH_2 + H_2N- \longrightarrow -O(C=S)NH-$
$-OH + ClCH_2$–  $+ HO- \longrightarrow -OCH_2CH(OH)CH_2O-$ (+ H_2NH-) (NH–)
$-OH \longrightarrow -OCH_2(C=O)H + H_2N- + NaCNBH_3 \longrightarrow -OCH_2CH_2NH-$

Fig. 6. Chemical reactions for covalent modifications, grafting, and oligomer attachments to solid supports. The solid supports can be beads, membranes, or surfaces, with a starting functional group X. Functional group transformations can be carried out in a variety of ways (as needed) to provide group X* which represents one partner in the covalent linkage with group Y*. The Figure shows specifically the grafting of PEG, but the same repertoire of reactions can be used (however needed) to attach carbohydrates (with hydroxyl), linkers (with carboxyl), and/or DNA oligonucleotides and PNA oligomers that have been extended by suitable functional groups (amino or carboxyl). In some cases, group X* or Y* is pre-activated (isolable species from a separate reaction); alternatively, activation occurs in situ. Referring to PEG as drawn in the Figure, Y and Y* can be the same (homobifunctional) or different (heterobifunctional); in the latter case, (Y) can be protected for further control of the chemistry.

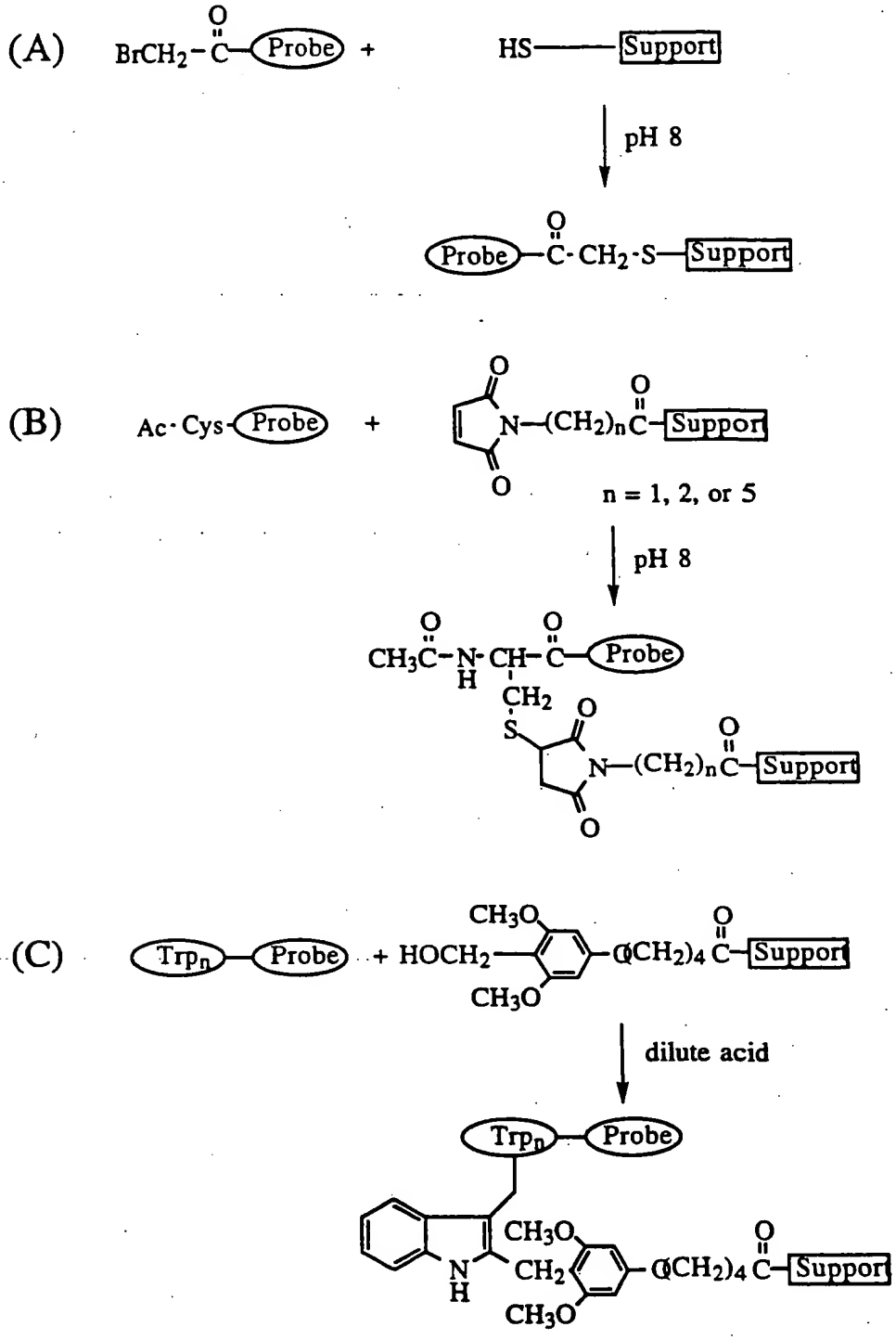


Fig. 7. Proposed chemistries for covalent attachment of DNA or PNA probes to solid supports. Chemically synthesized probes can be extended, on either end (shown here on the N-terminal of PNA or the 5' end of DNA). Further variations of the proposed chemistries are readily envisaged. (**A**) An amino group on the probe is modified by bromoacetic anhydride; the bromoacetyl function is captured by a thiol group on the support. (**B**) An N-acetyl, S-tritylcysteine residue coupled to the end of the probe provides, after cleavage and deprotection, a free thiol which is captured by a maleimido group on the support. (**C**) The probe contains an oligo-tryptophanyl tail (n = 1 to 3), which is captured after treatment of a HAL-modified support with dilute acid.

(iv) Immobilization of individually synthesized DNA oligonucleotides or PNA oligomers onto solid supports

(a) Synthesis of 24-mer DNA oligonucleotides and 24-mer PNA oligomers. The five sequences listed in Table 2 are designed for testing the hybridization properties of zip code arrays. They will be synthesized by Core B as DNA oligonucleotides using standard phosphoramidite chemistries [29], and incorporating an "aminolink" group at the 5'-terminus. In addition, the same sequences will be synthesized as PNA oligomers by stepwise Boc or Fmoc solid-phase chemistry, or by a segment condensation approach using suitably protected PNA tetramers. The PNA will have one endgroup blocked (e.g., acetyl on N-terminus, or amide on C-terminus), and the other terminus extended with ε-aminocaproic acid to provide a free aliphatic amino or carboxyl site for ultimate linking to the solid support. The complementary zip code oligonucleotide sequences will be prepared with a fluorescent "Fam" group at the 5'-end, by Core B.

Synthetic DNA oligonucleotides or PNA oligomers will be released from the resin supports, concurrent with removal of side-chain protecting groups. These modified oligomers will be purified to homogeneity by well-precedented polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC: reversed-phase or anion-exchange) procedures. Immobilization to solid supports will follow, as described below.

(b) Covalent attachment of DNA oligonucleotides or PNA oligomers to solid supports. The purified oligomers all contain a free aliphatic amino group at the terminus, which allows attachment to a derivatized membrane according to Zhang [40]. Other attachment chemistries based on amino group chemistry will also be pursued, building on a wealth of precedents for connecting functionalized polymers and proteins to each other and to solid matrices [54] (Fig. 6). These procedure can be carried out in series with several probes, resulting in site-specific attachments. Once the complementary zip code probes have been immobilized, oligonucleotide hybridizations using fluorescently labeled zip codes will be carried out to evaluate both capacity and signal to noise ratio, as described in Core B.

Encouraging results in the experiments outlined above would provide impetus to the exploration of additional immobilization ("capture") chemistries, which need to be rapid, specific, and non-destructive to the combination of functional groups found in DNA oligonucleotides and PNA oligomers. Our strategy involves incorporation, through synthesis, of alternative functional groups at either end of the probe, together with modification of the support by a suitable complementary functional group. More specifically, we can take advantage of the facile S-alkylation or Michael addition of thiol groups, or of the reaction in dilute acid of indole moieties with tris(alkoxy)benzyl carbocations (Fig. 7).

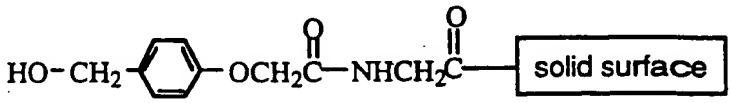
(v) Synthesis of oligonucleotides or PNA oligomers on solid supports and creation of arrays on solid surfaces

(a) Synthesis of 24-mer DNA oligonucleotides and 24-mer PNA oligomers on "shaved" beads. A second approach to constructing the arrays required for zip code capture starts with the assumption that suitable probes can be assembled and side-chain deprotected with covalent retention on beads used for solid-phase synthesis; these beads are then delivered to discrete addresses on a solid surface. General considerations have been outlined earlier; we consider this mode to represent a particularly pertinent case where application of our "shaving" concept may be critical to success. As before, the five sequences listed in Table 2 will be synthesized, either as DNA oligonucleotides (standard phosphoramidite chemistry) or as PNA oligomers (stepwise Boc or Fmoc chemistry).

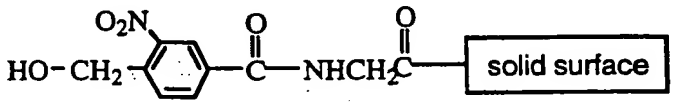
PEG-PS beads of 100μ diameter have a normal capacity of approximately 30 pmol, meaning that a shaved bead is predicted to hold about 0.1 to 0.5 pmol of final product. This level of material is well within the requirements of the subsequent hybridization studies. Chymotryptic "shaving" of a Boc-Trp-Gly-PEG-PS sequence generates a free α-amino group from "surface" glycine residues. For PNA synthesis, the C-terminal monomer is coupled to form a non-cleavable peptide bond; for DNA synthesis, an N-acetyl-serine spacer is introduced so that phosphoramidite synthesis begins off the free hydroxyl side-chain. For DNA chains upon completion of chain assembly, removal of the usual base-labile side-chain and phosphate protecting groups with aqueous ammonia yields the free probe oligomer linked covalently to the outside

areas of PEG-PS. For PNA oligomers, benzyloxycarbonyl-type protecting groups will be removed with strong acid, e.g., trifluoromethanesulfonic acid (the strategy may change if/when milder protection schemes under development at Millipore or by us are established).

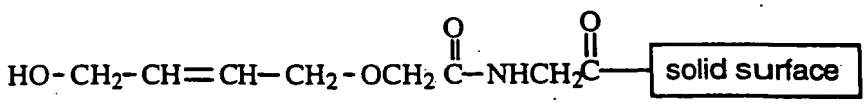
In order to better document the chemistry of chain assembly on "shaved" as well as control beads, the synthesis outlined above can be modified by introduction of base-stable, orthogonally cleavable linkers to separate the free glycine from the 3' or C-terminal residue. Suitable choices (Fig. 8) include acid-labile *p*-alkoxybenzyl (PAB), photolabile *o*-nitrobenzyl (ONb), or Pd(0)-cleavable allyl (Al) [47, 48]. The oligonucleotide or PNA products can be released from the support in a discrete step, and evaluated by standard analytical criteria as well as solution hybridization with the complementary sequences.



PAB



ONb



Al

Fig 8. Handles for attachment of oligopolymers to "shaved" beads. Handles are coupled through their carboxyl groups to "shaved" beads. The free hydroxyl on the left side of each structure can be esterified with the C-terminal PNA monomer or phosphitylated with a nucleoside phosphoramidite.

(b) Attachment of beads to solid surfaces. DNA or PNA synthesis on PEG-PS using automated instrumentation is generally carried out with several hundred thousand to several million beads, setting the stage for economical mass production of arrays. Each 200μ square pixel will contain several 100μ beads, and different spatial addresses will contain different beads. Several of our industrial collaborators are actively pursuing concepts for attaching beads to solid surfaces in a rapid and automated manner. (Please see letters of collaboration from Dr. James Coull of Millipore and Dr. Ronald Cook of Siris Labs). Briefly, these concepts include precise positioning of the bead over a gridded surface and melting one surface to attach them, using grooves or dimples in the surface to help position beads (using vacuum suction to guide a bead into a particular position), and/or projecting the beads onto a surface containing glue. For academic purposes, we will place beads onto a thin layer of some bonding material, such as epoxy. All bonding materials will need to be tested for resistance to high temperatures/high salt conditions, and to confirm the absence of non-specific binding to DNA oligonucleotides. Oligonucleotide hybridizations using fluorescently labeled complementary zip codes will be used to evaluate both capacity and signal to noise ratio as described in Core B.

(c) Variations. As necessary, the evolving literature methods for simultaneous syntheses of peptides or oligonucleotides at defined positions will be adapted in concert with the chemistry (linkers, protection strategies) and other concepts (shaving) presented above for beads. For example, it will be of interest to learn whether shaving of membranes, PEG-modified polyethylene surfaces, or pins helps with synthesis and/or hybridization efficiency. The various literature protocols for multiple synthesis are quite labor-intensive, but they may need to be pursued should we be unable to devise successful way to glue beads that contain DNA or PNA probes to surfaces, and to apply such materials for hybridization.

(vi) Direct synthesis of PNA arrays by masking/segment condensation on solid supports

(a) Perspective. In principle, arrays can be constructed most effectively by use of highly accurate masking and unmasking technology with per cycle yields of close to 100%. Unfortunately, current chemistries proceed in at best 97% yield per step, with a possible further drop-off as chain length increases. These relatively low efficiencies allow for construction of modest arrays in the octamer to decamer size range (even so, with substantial synthetic error rates which translate to hybridization at false addresses), but preclude construction of 24-mers needed as complementary zip codes in the cancer detection scheme of this program project proposal.

In the following, we propose a novel way to circumvent the aforementioned problems. The design of zip code arrays (in which individual arrays have substantial differences to minimize any chances of cross-reactivity; see earlier Fig. 5 and Table 1, and accompanying discussion) has been *integrated* with the synthetic strategy. Rather than carrying out stepwise synthesis to introduce bases one at a time, we use protected PNA tetramers as building blocks. (As shown below, these are easy to prepare; the corresponding protected oligonucleotide intermediates would require additional protection of the internucleotide phosphate linkages.) Construction of the 24-mer at any given address requires only six synthetic steps, with a likely improvement in overall yield by comparison to stepwise synthesis. Moreover, since failure sequences at each address are shorter and lacking at least four bases, there is no risk that these will interfere with correct hybridization or lead to incorrect hybridizations. This insight also means that "capping" steps will not be necessary.

Masking technology will allow several addresses to be built up simultaneously, as is explained below. As direct consequences of the manufacturing process for the arrays, several further advantages are noted. Each 24-mer address differs from its nearest 24-mer neighbor by three tetramers, or at least 6 bases. At low salt, each base mismatch in PNA/DNA hybrids decreases the melting temperature by 8°C. Thus, the T_m for the correct PNA/DNA hybridization is at least 48°C higher than any incorrect hybridization. Also, neighboring 24-mers are separated by 12-mers, which do not hybridize with anything and represent "dead" zones in the cancer detection profile. Finally, by choosing PNA addresses, we create rugged, reusable arrays.

The remaining description indicates methods for preparation of 36 unique PNA tetramers, and shows the mechanical/chemical strategy to prepare the arrays. Pilot experiments will result in the creation of a 5x5 array with 25 addresses of PNA 24-mers. Ultimately, all 36 tetramers can be incorporated to generate full-size arrays of 1,296 addresses.

(b) Synthesis of protected PNA tetramer building blocks. For each of the 36 unique sequences that have been designed (Table 1), we require the intermediates with appropriate protection on the α-amino group, on the side-chains, and with a free α-carboxyl group. This can be done readily by Fmoc chemistry on PAB or HAL resins, according to standard protocols published from our laboratory [50, 55]. Following cleavage in dilute acid, the protected intermediates will be purified by chromatography. Alternatively, we can use Boc chemistry with ONb (photolabile) or allyl (cleaved by Pd(0)) resins [47, 48, 56].

(c) Construction of PNA arrays. As stated already, only the pilot study with a 5x5 array is described. Considerations concerning the solid support are the same as described earlier: starting surfaces will contain free amino groups ("shaved" if necessary), a non-cleavable amide linkage will connect the C-terminus of PNA to the support, and orthogonal side-chain deprotection must be carried out upon completion of segment condensation assembly in a way that PNA chains are retained at their addresses. A simple masking device has been designed that contains 200μ spaces and 200μ barriers, to allow each of 5 tetramers to couple to the solid support in distinct rows (Fig. 7). After addition of the first set of tetramers, the masking device is rotated 90°, and a second set of 5 tetramers are added. This can be compared to putting icing on a cake as rows, followed by icing as columns. The intersections between the rows and columns will contain more icing, likewise, each intersection will contain an octamer of unique sequence. Repeating this procedure for a total of 6 cycles generates 25 squares containing unique 24-mers, and the remaining squares containing common 12-mers (Fig. 10).

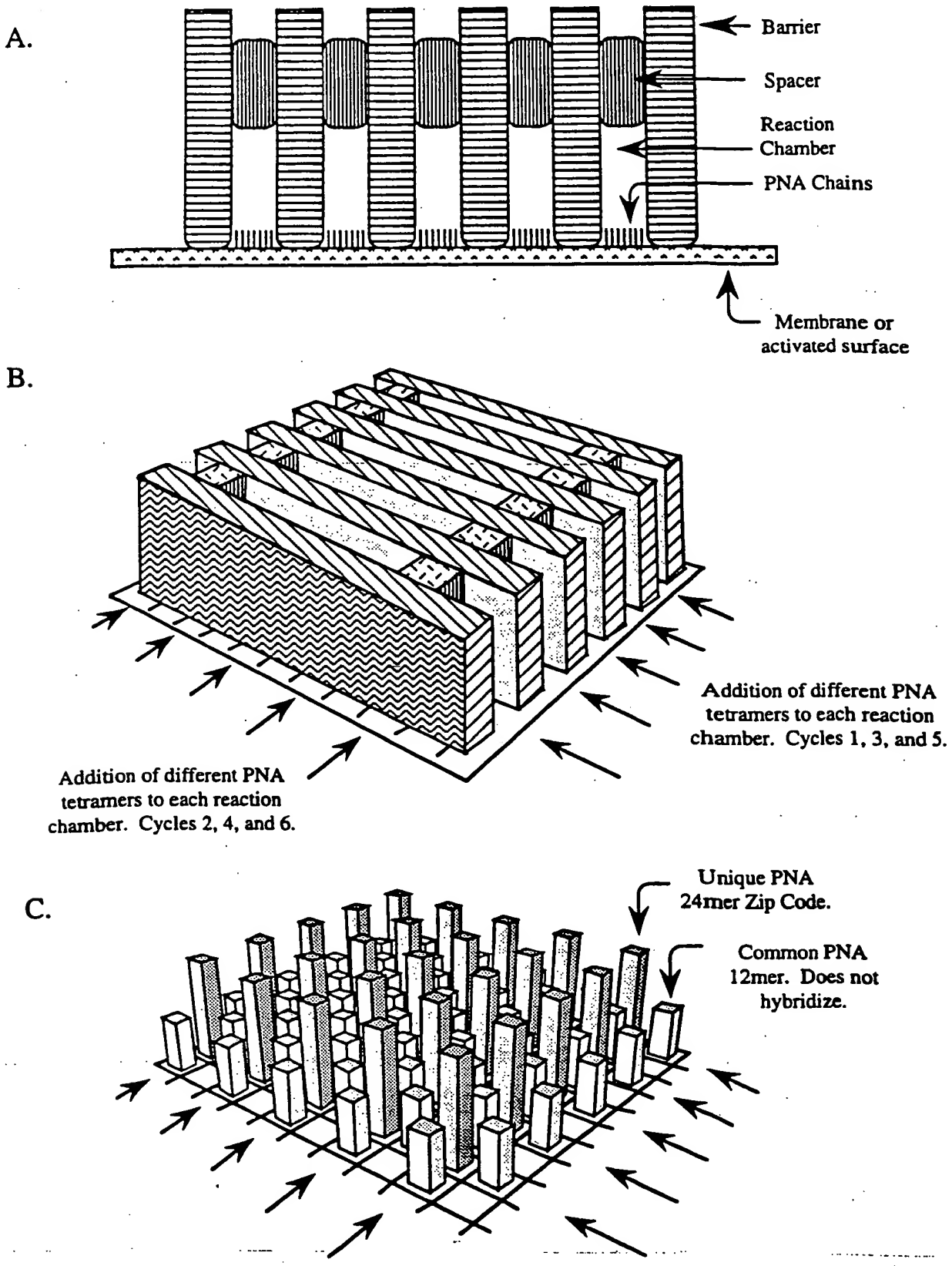


Fig. 9. Process for manufacturing an array (see following two pages for Fig. 10 and legends).

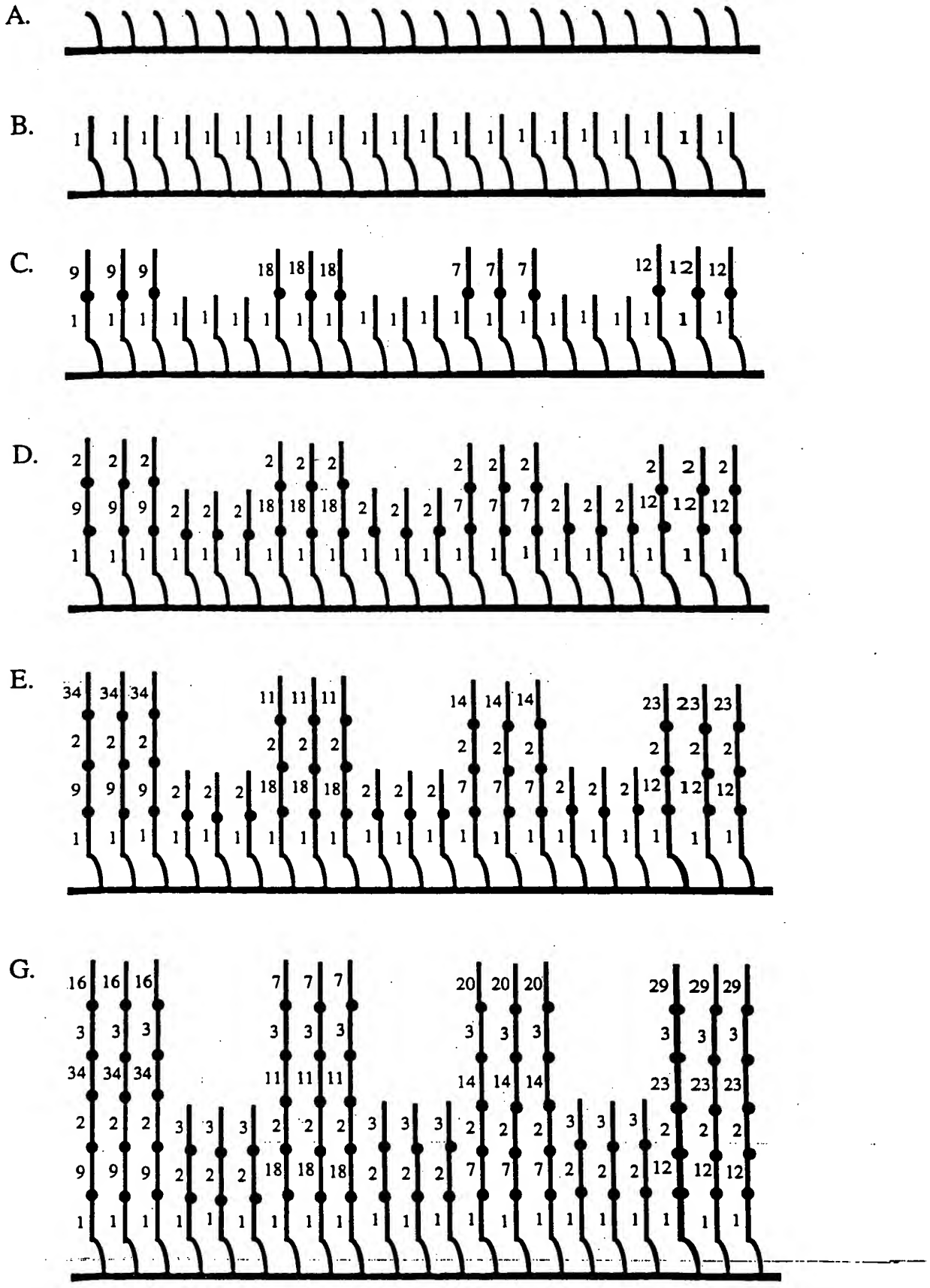


Fig. 10. Schematic cross-sectional view of synthesis of addressable array (see next page for legend).

Fig. 9. Process for manufacturing an array (legend). (A). Side view of reaction chambers. (B) Three-dimensional view of reaction chambers. Each wall and spacer is 100μ thick. These spacers form chambers of width 100μ. The multi-chamber device is pressed onto the membrane or activated solid surface, forming tight seals. The barriers may be coated with rubber or another material to avoid cross contamination from one chamber to the next. One must also make sure the membrane or solid support surface is properly wetted by the solvents. The membrane can be in a vertical position with a plate on each side to clamp the multi-chamber device to the membrane. Solvents are introduced at the bottom, rise up the chamber, and are removed from the top, much like an ABI four channel DNA synthesizer, except now there are 36 chambers and 36 different tetramer bottles. One proceeds by activating the surface, deprotecting, and adding a tetramer. The chamber is unclamped, the membrane is rotated 90°, and reclamped. A second round of tetramers are added. (C) Schematic bird's eye view of PNA oligomer array after completion of all 6 rounds of synthesis. Each tower represents 100 fmole of oligomers. Taller towers represent full size 24-mers which result from 6 rounds of synthesis in alternating directions. Each 24-mer tower represents a unique PNA sequence. Smaller towers represent half-size 12-mers which result from 3 rounds of synthesis in the same direction. All smaller towers in the same row as the arrows are of identical sequence. For clarity, the towers have been drawn as individual units, even though in the "real" synthesis they will be the same dimension as the grid squares and thus appear fused to each other. A "side view" of these individual towers is shown in Fig. 10.

Fig. 10. Schematic cross-sectional view of synthesis of addressable array (legend). (A) Attachment of flexible spacer (linker) to surface of array. (B) Synthesis of the first rows of PNA tetramers. Only the first row, containing tetramer 1, is visible. The multi-chamber device is placed so that additional rows, each containing a different tetramer, are behind the first row. (C) Synthesis of the first columns of PNA tetramers. The multi-chamber device has been rotated 90°. Tetramers 9, 18, 7, and 12 were added in adjacent chambers. (D) Second round synthesis of the PNA rows. The first row contains tetramer 2. (E) Second round of synthesis of PNA columns. Tetramers 34, 11, 14, and 23 were added in adjacent chambers during the second round. (F) (Not shown) Third round synthesis of PNA rows. The first row contains tetramer 3. (G) Structure of array after third round synthesis of columns, adding tetramers 16, 7, 20, 29. Note that all 24-mer PNA oligomers within a given row or column are unique, hence achieving the desired addressable array. Since each 24-mer differs from its neighbor by three tetramers, and tetramer differs from each other by at least 2 bases, then each 24-mer differs from the next by at least 6 bases. Each mismatch significantly lowers T_m, and the presence of 6 mismatches in just 24 bases would make cross hybridization unlikely even at 35°C. Note that the smaller 12-mer sequences are identical with one another, but are not at all common with the 24-mer sequences. Even though the particular 12-mer sequence may be found within a 24-mer elsewhere on the grid, for example 17-1-2-3-28-5, a zip code will not hybridize to the 12-mer at temperatures above 50°C.

Our design for a masking device is essentially the same as the masking technique developed by Maskos and Southern [33, 36]. This device will facilitate the desired array synthesis, and allow us to move on to testing zip code hybridization with Core B. The masking device will be designed and prepared by our industrial collaborators Dr. Ronald Cook of Siris Labs, and Dr. James Coull of Millipore. Simultaneously, we will also create, by hand, test arrays on membranes with aid of the Biorad dot blot apparatus containing individual microtiter wells.

E. PROGRAM ASPECTS

We are developing solid-phase methodology which will allow multiplex detection o oligonucleotide ligation products that are indicative of cancer mutations. Specific aims of this project (Project 5) are: (i) Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions. (ii) Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers. (iii) Demonstration of scope and limitations of zip code concepts.

The zip code approach, including the key needs for its experimental implementation, arose throug extensive discussions between F. Barany, R.P. Hammer, and G. Barany. The studies described in Project 5 that are directed towards solid support development and evaluation, with respect to either immobilization of pre-synthesized oligomers or their direct synthesis, will interface closely with efforts of Core B to prepare the needed DNA and PNA primer, zip code, and complementary zip code sequences, and to carry out hybridization assays. A collaboration with Project 3 will provide 5-propynyl-uridine monomers for incorporation into either DNA or PNA that may have improved thermodynamic parameters in hybridization. Progress in Project 5 on the preparation and application of spatially addressable arrays to detection of LDR products will have an immediate impact on the cancer work described by Project 1 and 2, since it will then

be possible to test clinical samples at a significantly enhanced throughput. We also anticipate considerable interactions between Project 5 and Core A for computer-aided design of zip code sequences.

Addressable array capture will eventually be the preferred method of identifying mutations. In our initial examination of the p53 gene in colon, lung, and breast tumor samples we will only look for nine different mutations, V157, R175, H179, C242, G245, R248, R249, R273 and R282 (See Projects 1 and 2). By synthesizing LDR primers with tails of varying lengths we can easily distinguish between these mutations using gel or capillary electrophoresis. However, increasing the number of assayable mutations eventually makes electrophoretic detection less feasible. This is due to two reasons. First, mutation-specific LDR primers should differ in length by two bases for their products to be distinguished by electrophoresis. For a large number of mutations to be assayed together, very long primers would have to be synthesized. Second, only one mutant signal would be expected for most reactions. Since mutant signals will differ from each other by only two bases, minor defects in a gel lane could cause a misreading of the LDR product length and incorrect identification of the mutation. Both of these problems are currently overcome in our laboratory by using more than one color fluorescent label and internal standards within the same lane. However, once the primers become very long (75-100 bases), failure sequences (n-1, n-2) become increasingly harder to separate by HPLC or gel purification. In contrast, by synthesizing a unique 24 base zip code sequence to each LDR primer, the product can be captured by its complementary zip code at a discrete "address" on a two-dimensional array. Failure sequences do not present a problem for either the zip code sequence or its complementary address. A fluorecent signal at a specific address, as opposed to a specific size, thus indicates the presence of a specific cancer mutation. (See Core B.)

A reusable addressable array with high capacity and excellent signal to noise specificity would be of benefit to several of our collaborators who need to detect large number of mutations. This will aid in the detection and identification of: hundreds of microorganisms by identifying 16s polymorphisms (Dr. Carl Batt), dozens of ß-lactamase mutations responsible for third generation ß-lactam resistance (Dr. Patrice Courvalin), epidemiological studies based on HIV polymorphisms (Dr. Olen Kew), dozens of polymorphisms in the E6 and E7 genes of high risk HPV strains (Dr. Saul Silverstein), multiple germline mutations in single gene disorders (Dr. Eric Hoffman, Dr. Perry White, and Dr. Emily Winn-Deen), and multiple somatic mutations in tumor suppressor genes and oncogenes (Dr. John Kovach, Dr. Michael Osborne, Dr. Basil Rigas, Dr. John Sninsky, Dr. Mark Sobel, Dr. Steven Sommer, and Dr. Thierry Soussi). Please see letters of collaboration in the overview section of this program project grant.

F. TIMETABLE

General: The various aims of this research will be pursued in parallel, with successful results in one arena providing impetus for progress on other aspects. The focus of Project 5 is the chemical synthesis of zip code DNA and PNA sequences, ideally in spatially addressable arrays, on appropriately optimized solid supports. As requisite materials and/or structures become available, they will be tested in relatively short order by Core 2. The list below follows a combination of descriptions in "Specific Aims" and in "Experimental Design and Methods."

Task 1. Design and optimization of zip code/address duplexes.

a. Synthesis of zip code sequences and their complements as DNA, followed by solution annealing studies. Months 1 to 12.
b. Synthesis of zip code sequences and their complements as PNA, followed by solution annealing studies. Months 7 to 24.
c. Synthesis of 5-propynyl-U monomers (Figures 3 and 4), synthetic incorporation into DNA and PNA, and solution annealing studies. Months 12 to 36.

Task 2. Development and evaluation of solid support materials compatible with chemical synthesis of DNA oligonucleotides and PNA oligomers, and compatible with subsequent hybridization reactions.

a. Studies with commercially available membranes and literature methods for immobilization of end-group modified DNA and PNA. Months 1 to 30.
b. Studies with "shaved" beads, which will be used for solid-phase synthesis of DNA. Months 1 to 18.
c. Modifications in our laboratories of surfaces, beads, or membranes with hydrophilic spacers such as heterobifunctional polyethylene glycol (PEG) and/or carbohydrates (see Figure 6 and accompanying discussion), and further studies. Months 12 to 48.
d. Development of novel chemistry for covalent immobilization of synthetic DNA or PNA (Figure 7). Months 6 to 30.
e. Preparation and segment condensation of protected PNA tetramer building blocks (including optimization of protection scheme and coupling conditions), to build up 24-mer complementary zip code sequences which will be released into solution following chain assembly. Months 12 to 48.
f. Segment condensation using protected PNA tetramer building blocks to build up 24-mer complementary zip code sequences which will be deprotected but retained on suitable solid supports for subsequent hybridization reactions. Months 36 to 60.

Task 3. Establishment of methodology for synthesis of spatially addressable arrays of DNA oligonucleotides and PNA oligomers.

a. Adaptation of commercially available membranes and literature methods for immobilization of end-group modified DNA and PNA, in tandem with spot methods and/or masking technology, to prepare and test relatively small arrays. Months 12 to 48.
b. Application of additional advances from Task 2 towards generation of spatially addressable arrays (e.g., "gluing" of "shaved" beads to solid surfaces, direct masking/segment condensation on solid supports as outlined in Figures 9 and 10). Months 12 to 60.

G. HUMAN SUBJECTS / VERTEBRATE ANIMALS: Not applicable

I. CONSULTANTS/COLLABORATORS: Project 5 represents a collaboration of Dr. George Barany (Principal Investigator), University of Minnesota, Dr. Robert Hammer, Louisiana State University, and Dr. Francis Barany, Cornell University, Medical College. In addition, we have excellent connections with leading industrial laboratories that are at the forefront of developing and commercializing methodologies for preparation of PEG-PS supports and functionalized membranes, PNA synthesis, and oligopolymer array construction. Specifically, we are collaborating with the team at Millipore led by Dr. James Coull, and a start-up company named Siris that is headed by Dr. Ronald Cook (see supporting letters). Recently, Dr. Michael Egholm, first author of several of the seminal papers on PNA, joined Millipore as a research chemist. Dr. Derek Hudson, a long-time collaborator of Dr. George Barany (several joint publications), is currently at Siris. Letters and Biographical Sketches for collaborators are attached in the overview section of this program project grant.

J. CONSORTIUM/CONTRACTUAL ARRANGEMENTS: Please see following page.

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STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

Date: January 26, 1994

Grant Number: P01-

P-01 Application Title: PROGRAM PROJECT: NEW METHODS FOR CANCER DETECTION

Project # 5; DESIGN AND SYNTHESIS OF DNA AND PNA ARRAYS.

Proposed Project Period: Year 01; 12/01/94- 11/30/95

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grant policy and will establish the necessary inter-institutional agreement(s) consistent with that policy."

CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NY

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Principal Investigator:

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K. LITERATURE CITED.

1. Merrifield, R.B., *Solid-phase peptide synthesis.* J. Am. Chem. Soc., 1963. **85**: p. 899-904.

2. Barany, G. and R.B. Merrifield, *Solid-Phase Peptide Synthesis,* in *The Peptides,* E. Gross and J. Meienhofer, Editor. 1979, Academic Press: New York. p. 1-284.

3. Merrifield, R.B., *Solid phase synthesis.* Science, 1986. **232**: p. 341-347.

4. Barany, G., N. Kneib-Cordonier, and D.G. Mullen, *Solid-phase peptide synthesis: A silver anniversary report.* Int. J. Peptide Protein Res., 1987. **30**: p. 705-739.

5. Kent, S.B.H., *Chemical synthesis of peptides and proteins.* Ann. Rev. Biochem., 1988. **57**: p. 957-989.

6. Atherton, E. and R.C. Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach.* 1989, Oxford: IRL Press.

7. Atherton, E., D.L.J. Clive, and R.C. Sheppard, *Polyamide supports for polypeptide synthesis.* J. Am. Chem. Soc., 1975. **97**: p. 6584-6585.

8. Smith, C.W., G.L. Stahl, and R. Walter, *Poly-N-acrylylpyrrolidine. A new resin in peptide chemistry.* Int. J. Peptide Protein Res., 1979. **13**: p. 109-112.

9. Atherton, E., *et al.*, *A physically supported gel polymer for low pressure, continuous flow solid phase reactions: Application to solid phase peptide synthesis.* J. Chem. Soc., Chem. Commun., 1981. : p. 1151-1152.

10. Arshady, R., *et al.*, *Peptide synthesis part 1: Preparation and use of polar supports based on poly(dimethylacrylamide).* J. Chem. Soc. Perkin Trans. I, 1981. p. 529-537.

11. Small, P.W. and D.C. Sherrington, *Design and application of a new rigid support for high efficiency continuous-flow peptide synthesis.* J. Chem. Soc., Chem. Commun., 1989. : p. 1589-1591.

12. Kanda, P., R.C. Kennedy, and J.T. Sparrow, *Synthesis of polyamide supports for use in peptide synthesis and as peptide-resin conjugates for antibody production.* Int. J. Peptide Protein Res., 1991. **38**: p. 385-391.

13. Bernatowicz, M.S., *et al.*, *Recent developments in solid phase peptide synthesis using the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group strategy,* in *Current Research in Protein Chemistry: Techniques, Structure, and Function,* J.J. Villafranca, Editor. 1990, Academic Press: San Diego.

14. Frank, R., *Spot-synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support.* Tetrahedron, 1992. **48**: p. 9217-9232.

15. Frank, R. and R. Döring, *Simultaneous multiple peptide synthesis under continuous flow conditions on cellulose paper discs as segmental solid supports.* Tetrahedron, 1988. **44**: p. 6031-6040.

16. Eichler, J., M. Beyermann, and M. Bienert, *Application of cellulose paper as support material in simultaneous solid phase peptide synthesis.* Collect. Czech. Chem. Commun., 1989. **54**: p. 1746-1752.

17. Lebl, M. and J. Eichler, *Simulation of continuous solid phase synthesis: Synthesis of methionine enkephalin and its analogs.* Peptide Res., 1989. **2**: p. 297-300.

18. Eichler, J., *et al.*, *Evaluation of cotton as a carrier for solid-phase peptide synthesis.* Peptide Res., 1991. **4**: p. 296-307.

19. Büttner, K., H. Zahn, and W.H. Fischer, *Rapid solid phase peptide synthesis on a controlled pore glass support,* in *Peptides-Chemistry and Biology: Proceedings of the Tenth American Peptide Symposium,* G.R. Marshall, Editor. 1988, Escom Science Publishers: Leiden, The Netherlands. p. 210-211.

20. Tregear, G.W., *Graft copolymers as insoluble supports in peptide synthesis,* in *Chemistry and Biology of Peptides,* J. Meienhofer, Editor. 1972, Ann Arbor Sci. Publ.: Ann Arbor, MI. p. 175-178.

21. Bayer, E. and W. Rapp, *Polystyrene-immobilized PEG chains: Dynamics and application in peptide synthesis, immunology, and chromatography,* in *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications,* J.M. Harris, Editor. 1992, Plenum Press: New York. p. 325-345.

22. Barany, G., *et al.*, *Novel polyethylene glycol-polystyrene (PEG-PS) graft supports for solid-phase peptide synthesis*. ed. C.H. Schneider and A.N. Eberle. 1993, Leiden, The Netherlands: Escom Science Publishers. 267-268.

23. Zalipsky, S., *et al.*, *Preparation and applications of polyethylene glycol-polystyrene graft resin supports for solid-phase peptide synthesis.* Reactive Polymers, 1994. **in press.**

24. Jung, G. and A.G. Beck-Sickinger, *Multiple peptide synthesis methods and their applications.* Angew. Chem. Int. Ed. Engl., 1992. **31**: p. 367-383.

25. Geysen, H.M., R.H. Meloen, and S.J. Barteling, *Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid.* Proc. Natl. Acad. Sci. U. S. A., 1984. **82**: p. 3998-4002.

26. Fodor, S.P.A., *et al.*, *Light-directed, spatially addressable parallel chemical synthesis.* Science, 1991. **251**: p. 767-773.

27. Cass, R., *et al.*, *Pilot, A new peptide lead optimization technique and its application as a general library method,* in *Peptides - Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium,* R.S. Hodges and J.A. Smith, Editor. 1994, Escom: Leiden, The Netherlands.

28. Lam, K.S., *et al.*, *A new type of synthetic peptide library for identifying ligand-binding activity.* Nature, 1991. **354**: p. 82-84.

29. Caruthers, M.H., *Chemical synthesis of DNA and DNA analogues.* Acc. Chem. Res., 1991. **24**: p. 278-284.

30. Garegg, P.J., *et al.*, *Nucleoside H-phosphonates. III. Chemical synthesis of oligodeoxyribonucleotides by the hydrogen phosphonate approach.* Tetrahedron Lett., 1986. **27**: p. 4051-4057.

31. Froehler, B.C., P.G. Ng, and M.D. Matteucci, *Synthesis of DNA via deoxynucleoside H-phosphonate intermediates.* Nucleic Acids Res., 1986. **14**: p. 5399-5407.

32. Uhlmann, A. and A. Peyman, *Antisense oligonucleotides: A new therapeutic principle.* Chem. Rev., 1990. **90**: p. 543-584.

33. Southern, E.M., U. Maskos, and J.K. Elder, *Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: Evaluation using experimental models.* Genomics, 1992. **13**: p. 1008-1017.

34. Maskos, U. and E.M. Southern, *Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotides synthesised in situ.* Nucleic Acids Research, 1992. **20**: p. 1679-1684.

35. Maskos, U. and E.M. Southern, *Parallel analysis of oligodeoxyribonucleotide (oligonucleotide) interactions. I. Analysis of factors influencing oligonucleotide duplex formation.* Nucleic Acids Res., 1992. **20**: p. 1675-1678.

36. Maskos, U. and E.M. Southern, *A study of oligonucleotide reassociation using large arrays of oligonucleotides synthesised on a glass support.* Nucleic Acids Res., 1993. **21**: p. 4663-4669.

37. Fodor, S.P.A., *et al.*, *Multiplexed biochemical assays with biological chips.* Nature, 1993. **364**: p. 555-556.

38. Khrapko, K.R., *et al.*, *A method for DNA sequencing by hybridization with oligonucleotide matrix.* J. DNA Seq. Map., 1991. **1**: p. 375-388.

39. Van Ness, J., *et al.*, *A versatile solid support system for oligodeoxynucleoside probe-based hybridization assays.* Nucleic Acids Res., 1991. **19**: p. 3345-3350.

40. Zhang, Y., *et al.*, *Single-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides.* Nucleic Acids Res., 1991. **19**: p. 3929-3933.

41. Egholm, M., *et al.*, *Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone.* J. Am. Chem. Soc., 1992. **114**: p. 1895-1897.

42. Egholm, M., *et al.*, *Recognition of guanine and adenine in DNA by cytosine and thymine containing peptide nucleic acids (PNA).* J. Am. Chem. Soc., 1992. **114**: p. 9677-9678.

43. Egholm, M., *et al.*, *Peptide nucleic acids containing adenine or guanine recognize thymine and cytosine in complementary DNA sequences.* J. Chem. Soc., Chem. Commun., 1993. : p. 800-801.

44. Egholm, M., *et al.*, *PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules.* Nature, 1993. **365**: p. 566-568.

45. Christensen, L., *et al.*, *Improved synthesis, purification, and characterization of PNA oligomers,* in *Innovation and Perspectives in Solid Phase Synthesis and Complementary Technologies: Biological and Biomedical Applications - 1994,* R. Epton, Editor. 1994, SPCC (UK) Limited:

46. Vagner, J., *et al.*, *Novel methodology for differentiation of "surface" and "interior" areas of polyoxyethylene-polystyrene (POE-PS) supports: Application to library screening procedures,* in *Innovation and Perspectives in Solid Phase Synthesis and Complementary Technologies: Biological and Biomedical Applications - 1994,* R. Epton, Editor. 1994, SPCC (UK) Limited:

47. Fields, G.B., Z. Tian, and G. Barany, *Principles and Practice of Solid-Phase Peptide Synthesis,* in *Synthetic Peptides: A User's Guide,* G. Grant, Editor. 1992, W.H. Freeman and Co.: New York. p. 77-183.

48. Barany, G. and F. Albericio, *Recent progress on handles and supports for solid-phase peptide synthesis,* in *Peptides-Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium,* R.S. Hodges and J.A. Smith, Editor. 1994, Escom Science Publishers: Leiden, The Netherlands. p. in press.

49. Albericio, F., *et al.*, *Preparation and application of the 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle for the solid-phase synthesis of C-terminal peptide amides under mild conditions.* J. Org. Chem., 1990. **55**: p. 3730-3743.

50. Albericio, F. and G. Barany, *Hypersensitive acid-labile (HAL) tris(alkoxy)benzyl ester anchoring for solid-phase synthesis of protected peptide segments.* Tetrahedron Lett., 1991. **32**: p. 1015-1018.

51. Froehler, B.C., *et al.*, *Oligonucleotides containing C-5 propyne analogs of 2'-deoxyuridine and 2'-deoxycytidine.* Tetrahedron Lett., 1992. **33**: p. 5307-5310.

52. Harris , J.M., ed. *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications.* 1992, Plenum Press: New York.

53. Lofas, S. and B. Johnsson, *A novel hydrogel matrix on gold surface plasma resonance sensors for fast and efficient covalent immobilization of ligands.* J. Chem. Soc., Chem. Commun., 1990. : p. 1526-1528.

54. Goodchild, J., *Conjugates of oligonucleotides and modified oligonucleotides: A review of their synthesis and properties.* Bioconjugate Chem., 1990. **1**: p. 165-187.

55. Kneib-Cordonier, N., F. Albericio, and G. Barany, *Orthogonal solid-phase synthesis of human gastrin-I under mild conditions.* Int. J. Peptide Protein Res., 1990. **35**: p. 527-538.

56. Barany, G. and F. Albericio, *A three-dimensional orthogonal protection scheme for solid-phase peptide synthesis under mild conditions.* J. Am. Chem. Soc., 1985. **107**: p. 4936-4942.